Novel Methods for the

Ribosomal Incorporation of β-Amino Acids

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ABSTRACT

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Protein-protein interactions (PPIs) dominate all cellular functions across every domain of life. If PPIs become aberrant, they may result in many human diseases, such as cancer or Alzheimer's. Despite their clinical significance, modulating aberrant PPIs is a daunting task. Most PPI surfaces are long, hydrophilic and structurally complex. Thus, finding molecules that moderate specific aberrant PPIs is an important goal in drug discovery research. For example, PPIs have been modulated by peptidomimetics, synthetic peptides that assume three-dimensional structures similar to proteins, but unlike natural peptides, they are proteolytically stable. However, building libraries of peptidomimetics is challenging as current methods rely on solid phase peptide synthesis, which limits the size and diversity of peptidomimetic libraries. As such, using the translation machinery to synthesize peptidomimetics is an attractive approach.

In **Chapter 1**, we begin by discussing bacterial protein synthesis. Then, we delve into a detailed discussion of the application of the bacterial translation machinery for the *in vitro* translation of synthetic peptides. In this discussion, we review the different technologies, their advantages and limitations with respect to the incorporation of amino acids with unnatural backbones.

After reviewing the methods used to incorporate backbone analogs, and their compatibility with the bacterial translation machinery, we describe a novel approach for the ribosomal incorporation of β-amino acids analogs containing an α-substituent, α-hydroxy-β-amino acids (**Chapter 2**). We demonstrate that the ribosome incorporates this new class of substrates through the formation of an intermediate ester bond that rapidly rearranges to form a native peptide bond. Using this approach, we show that α-hydroxy-β-amino acid single incorporation efficiencies are comparable the incorporation efficiencies obtained with natural amino acids.

In **Chapter 3**, we apply this approach to the synthesis of peptides containing multiple α-hydroxy-β-amino acids. This chapter describes the results obtained with the *in vitro* synthesis of peptides containing two consecutive α-hydroxy-β-amino acids, three consecutive α-hydroxy-β-amino acids, and alternating α-hydroxy-β-amino acids and α-amino acids. Based on these results, we propose experiments to improve

these incorporation yields for the application of this technology for the *in vitro* synthesis of diverse peptidomimetic libraries.

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aaRS	aminoacyl-tRNA synthetase
aa-tRNA	aminoacyl-tRNA
A-site	aminoacyl-tRNA binding site
Ala	alanine
Arg	arginine
AMP	adenosine monophosphate
ATP	adenosine triphosphate
CME	cyanomethyl ester
Cryo-EM	cryo-electron microscopy
Cys	cysteine
Daa	D-amino acid
D-aa-tRNA	D-aminoacyl-tRNA
DBE	3,5-dinitrobenzyl ester
DNA	deoxyribonucleic acid
DTT	dithiothreitol
Dra	*D. radiodurans*
E. coli	*Escherichia coli*
EDTA	ethylenediaminetetraacetic acid
EF-G	elongation factor G
EF-Ts	elongation factor Ts
EF-Tu	elongation factor Tu
eTLC	electrophoretic TLC
E-site	exit site
fMet	N-formyl-methionine
FPLC	fast protein liquid chromatography
et al.	et alia
GTP	guanosine triphosphate

h	hour
Hma	*Haloarcula marismortui*
HPLC	high-performance liquid chromatography
IF-1	initiation factor 1
IF-2	initiation factor 2
IF-3	initiation factor 2
LiCl	lithium chloride
Lys	lysine
M	moles per liter (molar)
mM	millimoles per liter (millimolar)
min	minutes
mL	milliliters
mmol	millimole
mRNA	messenger RNA
nM	nanomoles per liter (nanomolar)
NMR	nuclear magnetic resonance spectroscopy
PCR	polymerase chain reaction
P-site	peptidyl tRNA binding site
Phe	phenylalanine
PTC	peptidyl transferase center
PPI	protein-protein interaction
poly(U)	polyuridic acid
RNA	ribonucleic acid
RT	room temperature
TM	translation machinery
s	seconds
sec	seconds
TFA	trifluoroacetic acid

TLC	thin layer chromatography
TM	translation machinery
tRNA	transfer RNA
Tyr	tyrosine
UAA-tRNA	unnatural aminoacyl-tRNA
vs	versus
wt	wild type
μL	microliter
μM	micromoles/liter

To my parents, Luigi and Lucía, and grandmother, Milca, who always supported me in every step of the way in whatever way they could, and always reminded me that I am strong and can handle anything that's thrown in my way.

To Kelvin, your unwaivering support these past years made all the difference.

To Gian and Giuli, may this serve as a reminder that we are capable of overcoming much more than we think possible.

1 Chapter 1 Introduction

Protein-Protein Interactions (PPIs) are present in all domains of life

Protein-protein interactions (PPIs) are deliberate stable or transient interactions between two proteins that regulate many essential cellular processes in all domains of life. Decades of structural, biochemical, genetic and computational studies have revealed that PPIs are intimately linked to protein structure, which is a result of their ability to assemble to form primary, secondary, tertiary and quaternary structures.[1–3] Proteins are composed of a defined amino acid sequence connected by amide bonds. These amide bonds interact with each other to form stable secondary structures such as β-sheets and α-helices. Secondary structures encourage amino acid side-chains to interact with each other via non-covalent interactions, allowing proteins to adopt the three-dimensional structures that confer unique functions and enables specific PPIs. In some cases, proteins interact with each other to form multi-subunit complexes. Electrostatic interactions, hydrogen bonds, and hydrophobic interactions between subunits determine the quaternary structure of multi-subunit complexes. By studying protein structure and the role that protein structure plays in PPIs, we can begin to elucidate how specific PPIs regulate the fundamental intra- and extra-cellular processes that govern the growth and the fate of cells.[4] Among other things, PPIs may influence biological processes by promoting the activity of other proteins, by inactivating other proteins, and by changing the substrate specificity of a protein.[5] Notably, the deregulation of PPIs caused by mutations in the genes that encode proteins is frequently associated with abnormal phenotypes. In humans, abnormal phenotypes manifest as cancers, neurological diseases such as Parkinson's or Alzheimer's, and facile propagation of viral proteins by host cells. [6–13] Understanding the structural basis of PPIs that are implicated in human diseases will ultimately guide efforts to design drugs to target them.

Structural Features of PPIs

Structural studies are essential for drug discovery efforts because they facilitate the engineering of synthetic molecules that specifically target aberrant PPIs. Structural studies have shown that PPI interfaces feature large flat surfaces ranging from 1000-2000Å in length, and include complementary geometries between the proteins involved, hydrophobic cores, hydrophilic bridges, hydrogen (H)-bonds and electrostatic

interactions.[4] Consequently, most PPIs were deemed 'undruggable' because they lacked small hydrophobic pockets where small molecules can bind.[14] Once site-directed mutagenesis experiments revealed that small hydrophobic 'hot spots' have high contributions to the binding energy of PPIs,[15–18] computational advances as well as several methods for discovering small molecule inhibitors of PPIs, including fragment screening and molecular design, emerged.[19,20] In spite of these technological advances, PPI ligands are proteins and, as such, one would expect inhibitors to structurally resemble their targets. Natural peptides that mimic the surfaces of target proteins have been used to inhibit PPIs, i.e. the p53·hDM2 interaction, which is implicated in abnormal cell growth and replication, leading to tumors.[21] Natural peptides are attractive, potential PPI inhibitors large libraries of these peptides can be synthesized *in vitro* quickly and efficiently using the translation machinery (TM) in conjunction with *in vitro* display methods.[22] While effective, peptides with natural backbones are highly susceptible to proteolysis and do not retain their secondary structures in solution.[23] One way to address this problem is by using peptidomimetics, or synthetic peptides that mimic protein surfaces.[24] Peptidomimetics can be natural peptides that have been cyclized after *in vitro* synthesis,[25] or peptides with synthetic backbones (Figure 1.1).

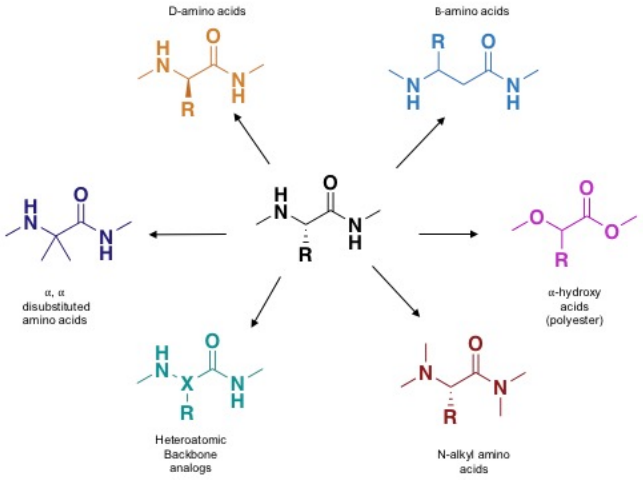

Figure 1.1 Types of Peptidomimetics

Modifications to the peptide backbones confer increased proteolytic stability as well as higher binding affinities for their target protein. Ideally, we large libraries of peptidomimetics would be synthesized using the TM. Unfortunately, the limited number and type of monomers that the ribosome can use greatly limits the structural diversity of these libraries. Hence, developing new technologies for the synthesis of unnatural peptides represents an important goal. In this chapter, I will give a brief overview of the TM with an emphasis on how our understanding of the TM has facilitated the development of technologies for synthesizing synthetic polypeptides with unnatural backbones.

The translation machinery (TM)

The synthesis of proteins by the TM requires that the genetic information stored in the form of DNA is first transcribed into an intermediate messenger RNA (mRNA) template. Subsequently, the mRNA templates are decoded by the TM and translated into proteins Translating mRNA into protein depends on the genetic code, which specifies the set of three nucleotides, or codons, that correspond to a particular amino acid.[26] Since each codon is a triplet and there are four nucleotides, the genetic code contains 64 distinct codons (4^3 codons). Out of the 64 codons, 61 correspond to one of the 20 canonical amino acids.

An important step towards achieving accurate translation is the aminoacylation, or "charging" of the transfer RNA (tRNA) substrates that carry the amino acids to the ribosome, with their cognate amino acids. Each tRNA carries one amino acid, but one amino acid may be charged onto more than one tRNA; tRNA variants that carry the same amino acid are called isoacceptors.[27]

While tRNAs have varied primary structures, the tRNAs have a defined secondary and tertiary structure that help maintain the accuracy and fidelity of translation. The secondary structures of tRNAs is known as the cloverleaf model. The cloverleaf model arises from the stems that form at the portions of the primary sequence that are complementary, and the loops that form at the portions that lack complementary sequences.[28,29] It states that every tRNA contains 3 stem-loops: dihydrouridine stem-loop or D-arm (contains two modified bases, hydrouridine), TΨC stem-loop (contains one modified base, pseudouridine) and the anticodon stem-loop (AC-arm). They also have an acceptor stem, which is formed by the interaction between the 5' and 3' ends of the tRNA, and the variable loop, a small sequence that varies in length among different tRNAs. tRNAs also adopt a well-defined tertiary structure that resembles the letter L.[30,31] The L-

shape is due to base stacking that brings the D- and TΨC arms one on top of the other, and forces the acceptor stem and anticodon stem-loop to be in the opposite ends of the tRNA. Amino acids are esterified to the acceptor stem, while the anticodon dictates which amino acid is incorporated into a polypeptide chain in response to a codon.

The central component of the TM is the ribosome, a universally conserved macromolecular machine composed of two subunits that together form binding sites for the aa-tRNA substrates, the A-site, the P-site, and the E-site. In bacteria, the two subunits are the large, or 50S, subunit and the small, or 30S subunit. Each subunit is made of a ribosomal RNA (rRNA) backbone and a subset of ribosomal proteins that interact with the rRNA to stabilize its correct folded structure. In addition, the TM also includes the translation factors, which interact with the ribosome to ensure that protein synthesis proceeds with high efficiency, speed, and fidelity.

Translation occurs in three major steps termed the initiation, elongation and termination steps. The initiation step is facilitated by initiation factors (IF1, IF2 and IF3); the elongation step is facilitated by elongation factors (EF-Tu, EF-Ts and EF-G); and, the termination step is facilitated by release factors (RF1, RF2 and RF3). In this chapter, I will focus my discussion on the elongation step, as it is arguably the most important step for (UAA) amino acid incorporation.

During translation initiation, an elongation-competent 70S ribosomal complex is formed that contains the mRNA start codon and the initiator tRNA in the P site, and an empty A site. Elongation is a cyclical process that involves three main steps: aa-tRNA selection, peptide bond formation, and translocation. Below, I discuss these three steps of the elongation cycle in more detail.

Initial aminoacyl-tRNA selection and proofreading ensures translation fidelity

Initial selection happens when the EF-Tu:GTP:aa-tRNA ternary complex (TC), delivers the aa-tRNA to the ribosomal A site.[32] Initially, the aa-tRNA is bound in the so-called A/T hybrid state. In the A/T state, the aa-tRNA anticodon stem loop (ASL) is bound to the A site while the aa-tRNA acceptor stem remains bound to EF-Tu. [33,34] In the A/T hybrid state, the ribosome quickly screens the nature of the codon:anticodon interaction.[35,36] During this initial selection, if the codon:anticodon interaction is non-cognate (i.e. there are not complementary base pairs), the ternary complex falls off. During proofreading, the codon:anticodon

interaction is interrogated a second time. If the codon:anticodon interaction is near-cognate (i.e. the first or second position of the codon: anticodon interaction is not able to base pair, but the other two bases are cognate), then the ternary complex falls off. [37] If the aa-tRNA is cognate, that is to say that the codon:anticodon interaction results in three complementary base pairs or two complementary base pairs with a wobble pair in the third position, a series of rearrangements within the 50S are triggered by the codon:anticodon interaction.[33] GTP is hydrolyzed and EF-TU:GDP dissociates from the ribosome, and the aa-tRNA acceptor stem accommodates into the A site.[38] This elongation complex is now ready to participate in peptide bond formation. This process is visualized in Figure 1.2.

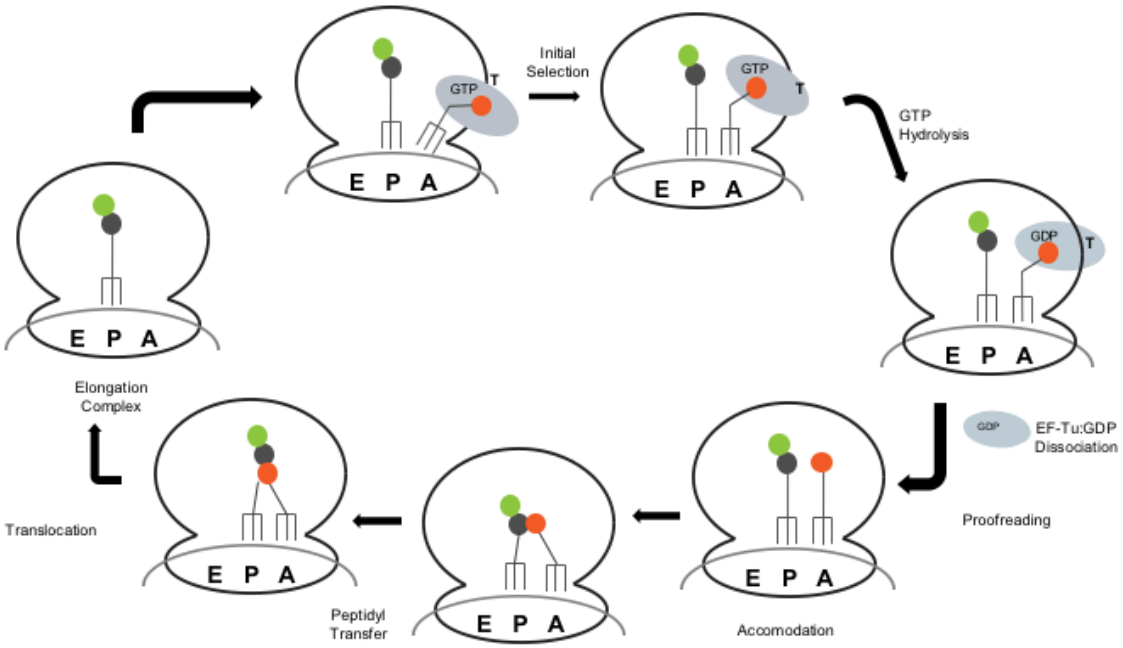

Figure 1.2 Elongation Cycle - Initial Selection and Proofreading

Peptide bond formation

The peptidyl transferase center (PTC) is located on the 50S subunit and it is responsible for catalyzing peptide bond formation (polymerization of amino acids into polypeptides). During peptide bond formation, the nucleophilic α-amine of the aa-tRNA in the A site attacks the carbonyl carbon of the peptidyl-tRNA in the P site, resulting in the formation of a tetrahedral intermediate. The tetrahedral intermediate then collapses yielding an amide bond that extends the peptide by a one amino acid, and transfers the peptide

chain to the A-site tRNA. Hence, during peptide bond formation, the P-site peptidyl-tRNA serves as the "peptide chain donor", and the A-site aa-tRNA serves as the "peptide chain acceptor" Figure 1.3.

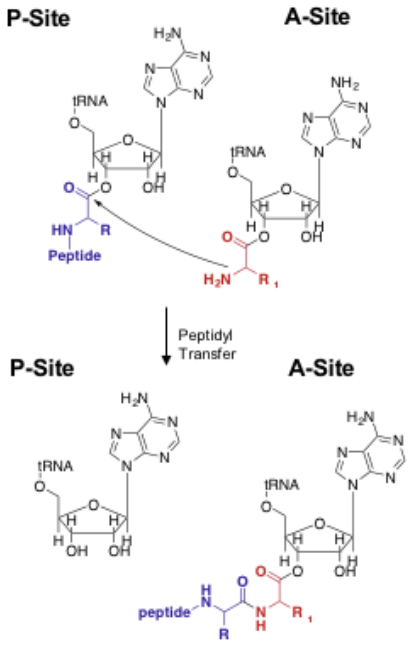

Figure 1.3 Peptidyl Transfer Reaction

Structural and biochemical studies elucidated the PTC's mechanism of action. Fragment reactions where small molecules that mimic aa-tRNAs, such as puromycin and puromycin analogs, react with the P-site substrate demonstrated that ribosome's catalytic activity is located on the 50S subunit. [39,40] Moreover, while the 50S subunit is composed of rRNA and ribosomal proteins, ribosomal proteins do not play a significant role in peptide bond formation.[41] When the first high-resolution crystal structure of the 50S subunit complexed to two aa-tRNA substrate analogs it shows that there are no proteins near the site on the PTC where the nucleophilic attack occurs. Therefore, the ribosome's catalytic activity is exclusively derived from rRNA, making the ribosome a ribozyme. [42,43] Further biochemical studies where the rates of tRNA accommodation was examined as a function of pH confirmed the PTC catalyzed peptide bond formation is pH independent suggesting that the ribosome catalyzes peptide bond formation by lowering the entropy of activation.[44,45]

Translocation

Translocation, the process in which the mRNA and the A-site and P-site tRNAs are shifted upstream to reveal a new codon in the A site, is catalyzed by EF-G. During elongation the ribosome oscillates between two states that are dictated by the position of the tRNA substrates. In the pre-translocation state (PRE), the peptidyl tRNA is bound to the A site and the deacylated tRNA is bound to the P site. In contrast, in the post-translocation state (POST), the peptidyl tRNA is bound to the P site and the deacylated tRNA is bound to the E site.[46,47] Interestingly, in the absence of EF-G:GTP, PRE-state ribosomal complexes have been shown to stochastically fluctuate between two global states (GSI and GSII).[34,48,49] In the first global state, the subunits are in the non-rotated conformation, the tRNAs are in the classical A/A and P/P configurations, and theL1 stalk is in the open conformation. In the second global state the small subunit platform rotates with respect to the large subunit, the L1 stalk adopts a closed conformation, and the peptidyl-tRNA in the A site and the deacylated tRNA in the P site are in hybrid A/P and P/E states, respectively. In these hybrid states, the ASLs of both tRNAs remain static on the small subunit, while the acceptor ends spontaneously move to the E site and the P site. [49,50] In the presence of EF-G:GTP, GSII is favored, which ultimately results in movement of the ASLs of the P/E and the A/P hybrid tRNAs to the E and P sites (Figure 1.4).[49]

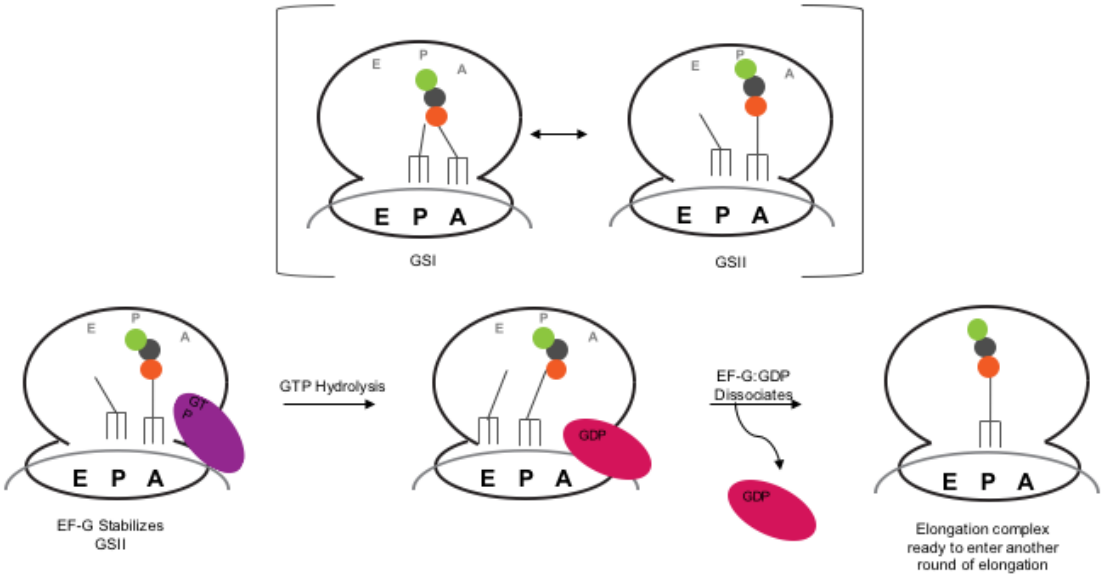

Figure 1.4 Translocation

Synthesizing genetically encoded unnatural peptides

Early research exploring the mechanism of mRNA translation by the ribosome led to the identification of the key co-factors and substrates of the translation pathway. The use of cell-free extracts as *in vitro* translation systems, first reported in 1958, demonstrated the importance of ATP and GTP. Cell-free translation extracts were also instrumental in the identification of the aa-tRNAs, then referred to as soluble RNAs (sRNAs), which were hypothesized to serve as the adaptor molecule during protein synthesis.[51] The adaptor hypothesis proposed that the tRNA adaptor molecules were the link between the genetic information and the amino acids incorporated by the ribosome, and suggested that the ribosome was oblivious to the identity of the amino acid on the tRNA.[52] Yet, testing the validity of the adaptor hypothesis required more control over the cell-free translation systems. Gaining control over the cell-free translation systems was achieved by incorporating synthetic mRNAs and chemically modified aa-tRNAs into the cell-free translation extracts.[53–56] The results of early studies using these cell-free translation extracts seemed to confirm the adaptor hypothesis. Indeed, it was observed that the codon:anticodon interaction directed the incorporation of amino acids, not the identity of the amino acid.[57] As such, this approach of chemically modifying aa-tRNAs for obtaining misacylated tRNAs opened the door to the

mRNA-directed natural amino acid misincorporation,[58] and incorporation of amino acids with modified side chains, and backbones. [59–62]

Meanwhile, cell-free extracts were also used to elucidate the mechanism of action of puromycin, an antibiotic derived from the bacterium, *Streptomyces alboniger*[39] that resembles the 3' end of aa-tRNAs and reacts with the peptidyl tRNA to inhibit *in vivo* and *in vitro* protein synthesis.[63,64] Because puromycin's amino acid moiety is linked to the nucleoside via an amide bond instead of an ester bond, it cannot be elongated and therefore, arrests translation. The ease of changing puromycin's aminoacyl moiety, compared to misacylating aa-tRNAs, along with the ease of setting up puromycin analog assays paved the way for the first studies of the plasticity of the ribosomal PTC for different amino acid backbone analogs. [40,65]

The first examples of using both chemically modified aa-tRNAs and puromycin analogs to study the ribosome's substrate specificity were presented by Rich and Fahnestock. Using the initiator N-formyl-methionyl-tRNA^{fMet} (fMet-tRNA^{fMet}) as the P-site substrate and α-hydroxy puromycin as the A-site substrate, they showed that the ribosome catalyzes ester bond formation nearly as efficient as it catalyzes amide bond formation.[66] Later, they expanded on this result by showing that the ribosome is also capable of polymerizing deaminated phenylalanyl-tRNA^{Phe} (Phe-lac-tRNA^{Phe}) in response to a synthetic poly(U) message.[62] In addition, they showed that the ribosome is capable of site-specifically introducing deaminated alanyl-tRNA^{Ala} (lac-tRNA^{Ala}) or Phelac-tRNA^{Phe} into the second or fifth position, respectively, of a natural mRNA encoding a hexapeptide fragment (fMet-Ala-Ser-Asn-Phe-Thr) of the viral coat protein R17 *in vitro.*[67]

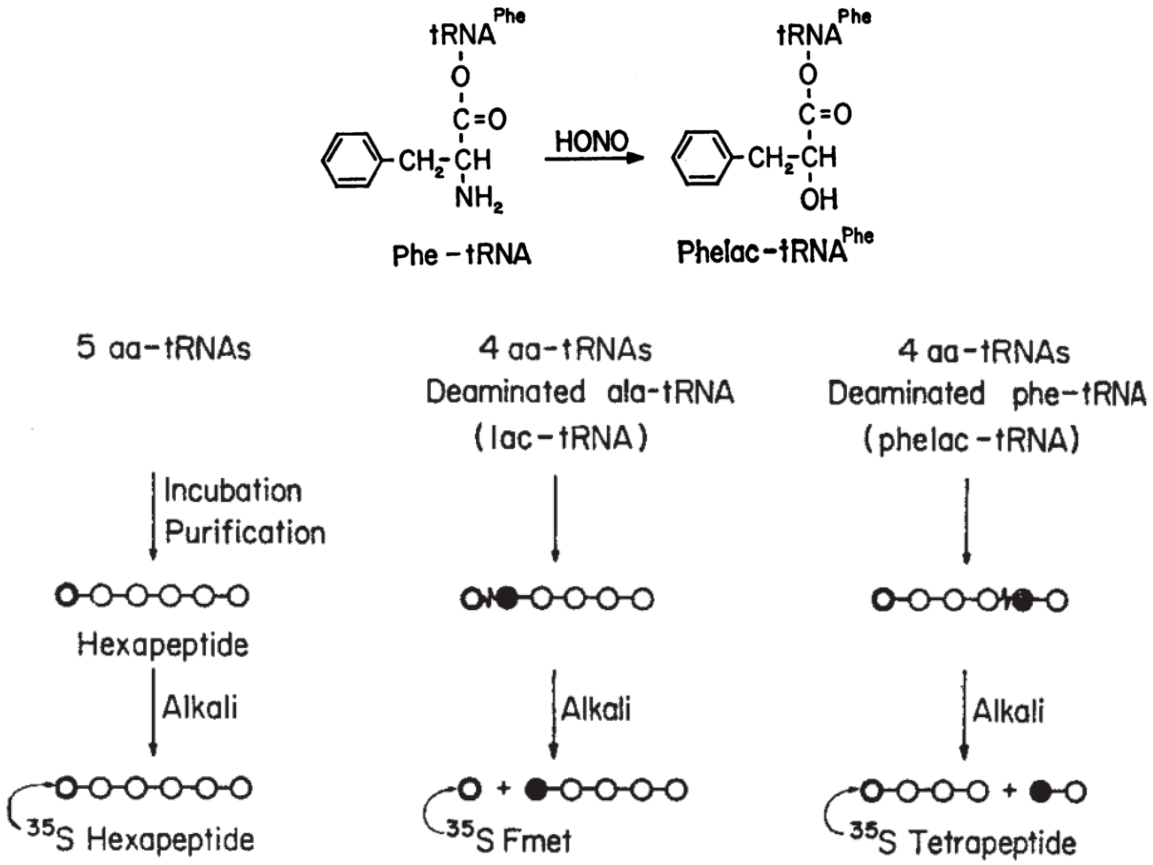

Figure 1.5 Ribosome-catalyzed ester formation[62, 67]

Subsequently, they showed that the co-incubation of thio-puromycin and N-acetyl-[^{3}H]-Phe-tRNA^{Phe} into cell-free translation extracts yielded a thioester, the identity of which was confirmed by co-migrating chemically synthesized authentic markers with the thioester translation product.[68] Translation reactions where the P-site puromycin analogs included either a thioester or a phosphinoester, suggest that the ribosome will accept heteroatoms in the P-site substrate. [69,70] However, it is not clear whether it will accept other heteroatom nucleophiles, other than NH_2, OH, and potentially SH.

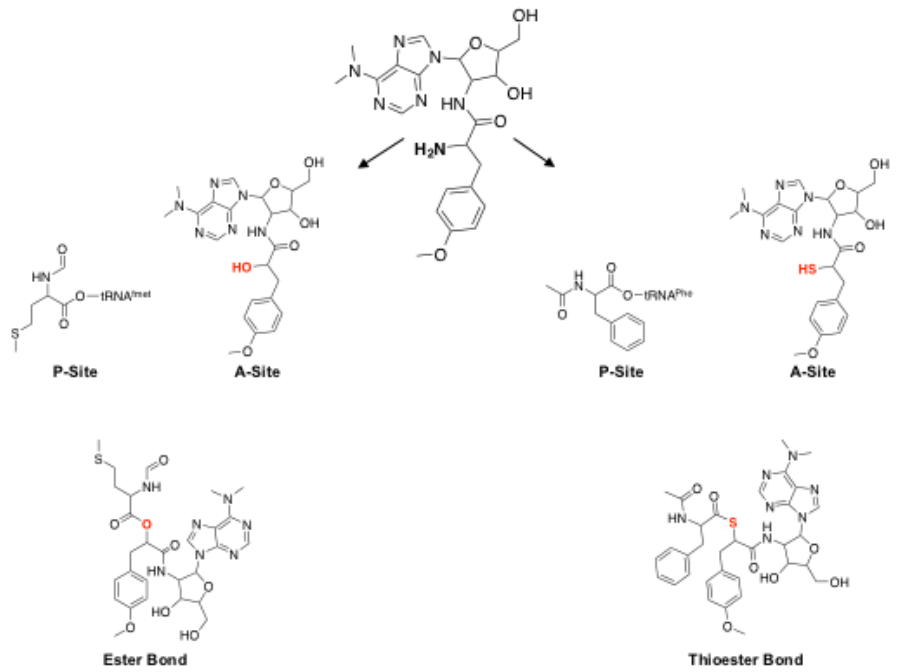

Figure 1.6 Ribosome-catalyzed ester and thioester formation[66,68]

Evolution of techniques for misacylating tRNAs

In nature, tRNAs are charged by aminoacyl synthetases (aaRSs). AaRSs are multi-domain enzymes that charge tRNAs, in a two-step reaction. In the first step, aaRSs bind and activate an amino acid using ATP as the energy source. The activation of the amino acid occurs when the amino acid carboxylate attacks the α-phosphate of the aaRS-bound ATP, resulting in the release of inorganic pyrophosphate and the formation of a high-energy aminoacyl adenylate (aa-AMP):aaRS complex. In the second step, the aa-AMP:aaRS complex binds to its cognate tRNA and catalyzes the esterification of the amino acid to either the 2'-hydroxyl or the 3'-hydroxyl of the terminal 3'-adenine (A76) of the tRNA.

The ability of aaRSs to both discriminate between different amino acids and to identify their cognate tRNAs is crucial for the accuracy of translation. It has been previously shown that in the absence of the isoleucyl-tRNA ($tRNA^{Ile}$), the Ile-aaRS can bind and activate valine (Val), forming an non-cognate Val-AMP:Ile-aaRS complex.[71] However, in the presence of $tRNA^{Ile}$, Ile-aaRS hydrolyzes Val-AMP rather than misacylting $tRNA^{Ile}$. In addition, if $tRNA^{Ile}$ is chemically misacylated with Val (Val-$tRNA^{Ile}$) and incubated

with Ile-aaRS, the Val-tRNA^{Ile} is readily deacylated.[72] These results reveal that aaRSs use pre-transfer and post-transfer editing mechanisms to ensure that tRNAs are charged with their cognate amino acids. Because of the high accuracy exhibited by aaRSs, chemical strategies were developed to misacylate tRNAs with various natural and unnatural amino acids.[73]

The chemical aminoacylation of tRNAs provided a universal platform to study the plasticity of the peptidyl transferase center of the ribosome using full-length, misacylated tRNAs. These studies, in turn, led to the development of new technologies for the ribosomal synthesis of peptides with unnatural backbones (discussed below). The first chemical synthesis of tRNAs harboring UAAs was reported by the Hecht group. Hecht and co-workers demonstrated that tRNAs could be misacylated with N-acetyl-UAAs, and that these UAAs were viable substrates for the ribosome. Using a standard ribosomal dipeptide formation assay.[74–76] they measured the ability of several misacylated tRNA^{Phe} variants including N-acetyl-D-Phe-tRNA^{Phe}, N-acetyl-DL-β-Phe-tRNA^{Phe}, and N-acetyl-D-Tyrosine (Tyr)-tRNA^{Phe} to function as peptide chain donors. Their results revealed that N-acetyl-DL-β-Phe exhibits remarkable donor activity, while N-acetyl-D-amino acids (D-aa's) do not, consistent with previous D-aa incorporation studies, where D-Tyr-tRNA^{Tyr} was charged by the natural Tyr-RS and incorporated into peptides using cell-free translation systems despite their low affinity for EF-Tu. [77,78] While these experiments represented the first time the structural plasticity of the PTC of the ribosome was probed, their use of UAA-tRNAs with blocked amines prevented them from studying the effect of these UAAs on binding of EF-Tu, binding to the ribosomal A site, and on their ability to act as peptide chain acceptors.

Further optimization of the synthetic approach for obtaining chemically misacylated tRNAs by various research groups expanded the application of chemical approaches to misacylate tRNAs. These new approaches made it possible to misacylate tRNAs with any free amino acids in high yields (~30-50% aminoacylation efficiency),[79,80] thereby permitting an investigation of the ability of D- and β-aminoacyl tRNAs to bind the A site and act at peptide chain acceptors. For example, protecting the α-amine of amino acids with an enzymatically cleavable protecting group afforded the first synthesis of D-Phe-tRNA^{Phe}, D-Tyr-tRNA^{Phe} and D,L-β-Phe-tRNA^{Phe} with free were α-amines.[81] Using the dipeptide formation assay, the acceptor ability of D-Phe-tRNA^{Phe}, D-Tyr-tRNA^{Phe} and D,L-β-Phe-tRNA^{Phe} was tested. Each UAA-tRNA was added independently to mRNA-programmed ribosomes containing N-acetyl-Phe-tRNA^{Phe} in the P-site

While D,L-β-Phe-tRNA^{Phe} is a good peptide chain donor, the results indicated that it is a poor peptide chain acceptor. D-aas were not incorporated.

Cell-Free Translation Assays and Nonsense Suppression

The development of a general method to misacylate tRNAs with UAAs led the Schultz lab to develop a strategy to site-specifically incorporate UAAs into proteins by mutating sense codons to the amber (TAG) stop codon.[82]

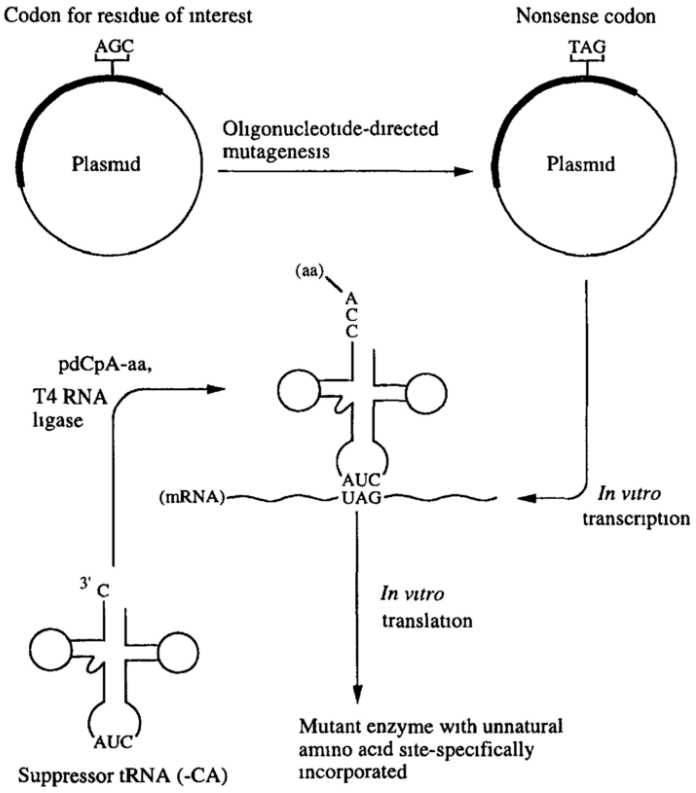

Figure 1.7 Site-directed mutagenesis

This method was used to synthesize variants of the T4 lysozyme (T4L) harboring UAAs to determine how these UAAs affect its stability.[83] Using a cell-free translation extract they investigated the ribosome's ability to incorporate cyclic and linear N-alkyl amino acids, α,α-disubstituted amino acids, α-hydroxy acids, D-amino acids and β-amino acids. Cyclic, linear N-alkyl amino acids and α,α-disubstituted amino acids are incorporated with suppression efficiencies ranging from 23-46%, while α-hydroxy acids are incorporated with a 30% suppression efficiency.[83] (Figure 1.8)

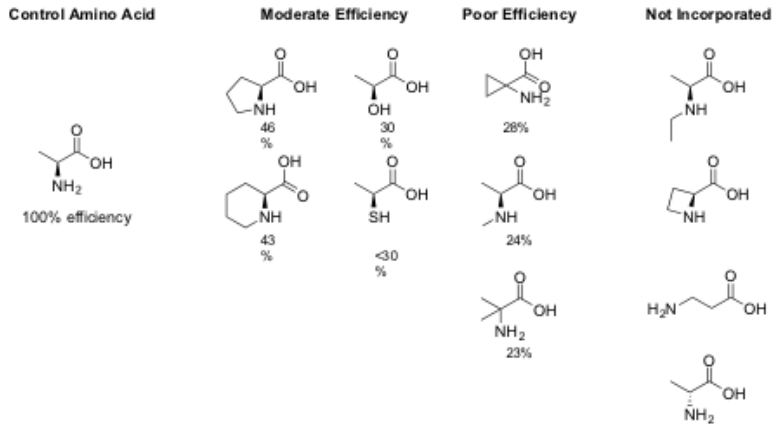

Figure 1.8 Backbone Analogs incorporated by site-directed mutagenesis

On the other hand, no suppression was observed when either D- or β-amino acids were used. Substituting natural amino acids with N-alkyl amino acids and α,α-disubstituted amino acids resulted in proteins with similar or slightly higher thermal stabilities.

The Chamberlain group developed a rapid assay to study the ability of UAAs to function as a peptide chain donor and as a peptide chain acceptor.[84,85] They misacylated a suppressor tRNA with UAAs and included them in their rapid assay.

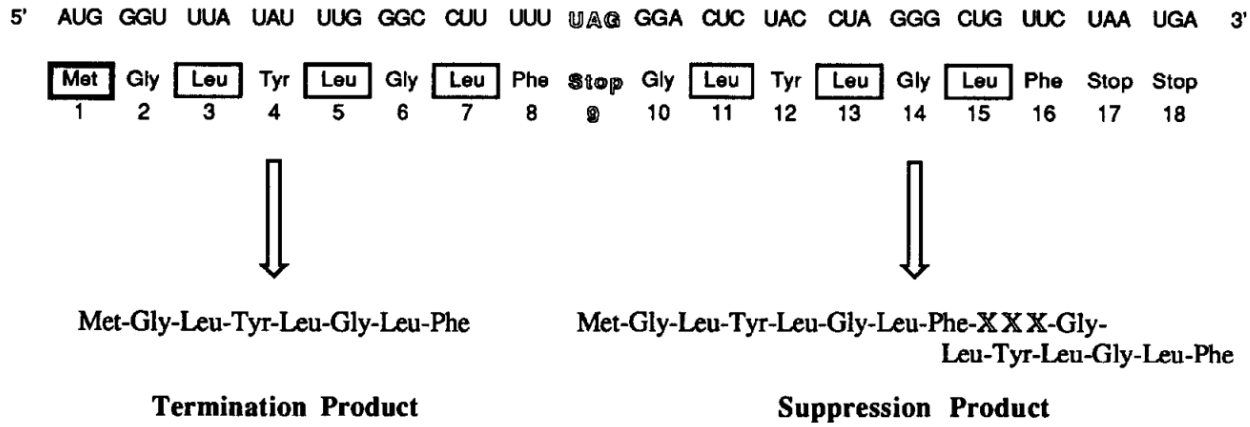

Figure 1.9 Rapid assay for stop codon read-through[84,85]

The rapid assay involves using an mRNA that codes for a 16-mer polypeptide encoding a stop codon in the ninth position. Successful stop codon suppression leads to the 16-mer polypeptide product, while unsuccessful suppression leads to a truncated 8-mer product. With this assay, they found that N-methyl-

Phe and α-hydroxy-Phe were incorporated but β-Phe-tRNA$_{CUA}$ and D-Phe-tRNA$_{CUA}$ were not. While these results are consistent with previous reports,[74,81] the authors never confirmed that the correct amino acid was being incorporated.

Cell-free translation extracts have also been used to study the ribosome's ability to synthesize dipeptides with altered connectivities.[86] To obtain peptides with an altered connectivity, N-chloroacetyl-Phe-tRNA^{Phe} was used as the P-site substrate. N-chloroacetyl-Phe-tRNA^{Phe} has two electrophilic carbons, as opposed to natural aa-tRNAs, which only have one – the carbonyl carbon. The A-site α-amine can react with the carbonyl carbon to give the expected dipeptide product or, alternatively, it could react with the N-chloroacetyl carbon, yielding a dipeptide with an altered connectivity.

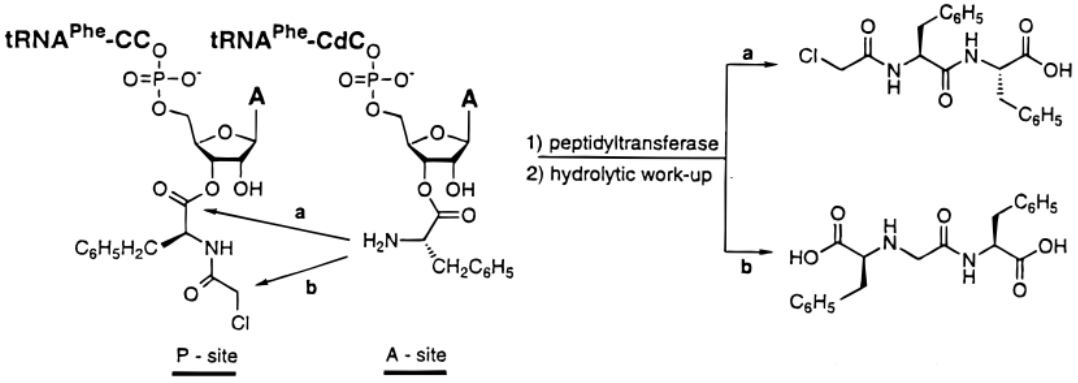

Figure 1.10 Ribosomal synthesis of peptides with altered connectivities[86]

Another way in which the peptide connectivity can be altered is by replacing the A-site aa-tRNA containing a natural amino acid with hydrazinophenylalanine-tRNA, which contains two nucleophiles, the α-amine and the β-amine.[87]

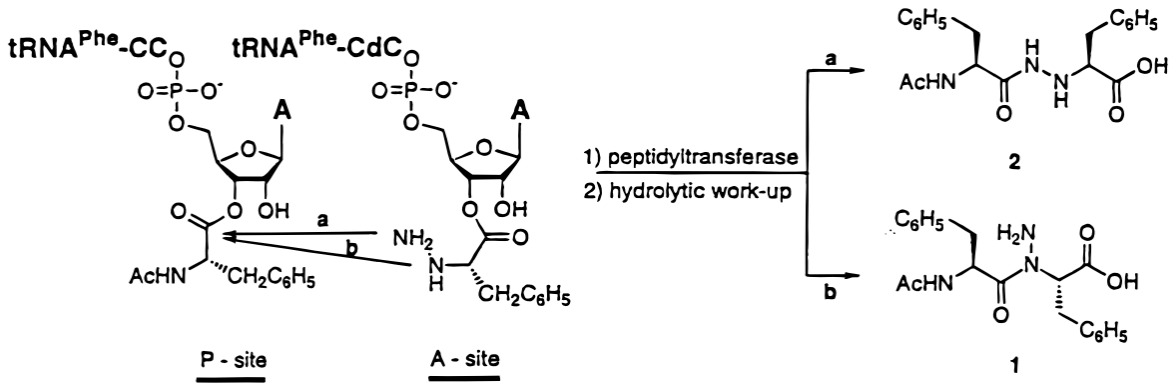

Figure 1.11 Dipeptide Formation with hydrazinophenylalanine[87]

Using a dipeptide assay, they showed that both the α-amine and the β-amine can act as nucleophiles as shown by the 71% dipeptide yield (Figure 1.11). HPLC analysis of the translation products indicated that the β-amine might be more reactive than the α-amine, as demonstrated by the ratios of 1:1.2 for α-amine product to β-amine product. Furthermore, when they incorporated this UAA into *E. coli* Dihydrofolate reductase (DHFR) using suppressor $tRNA_{CUA}$, they observed 9-15% stop codon read-through. These results are particularly interesting when you consider that β-amino acids do not seem to be compatible with the TM. It could be that the additional hydrogen bond donor on hydrazinophenylalanine enables this β-amino acid analog adopt a more stable configuration in the PTC when compared to β-amino acids with carbon backbones. The incorporation of hydrazinophenylalanine is further confirmed when they studied the ability of the suppressor $tRNA_{CUA}$ charged with (aminooxy)acetic acid and incorporated it into *E. coli* DHFR.[88] While they observed a low suppression activity (10%), their results seem to indicate that when part of the β-amino acid backbone is replaced with heteroatoms (N or O), incorporation of β-amino acid analogs is possible, providing a possible way to incorporate β-aa-tRNAs. Amber suppression in cell-free translation extracts have shown that β-hydroxy acids are also incorporated preferentially over β-amino acids, presumably, because β-hydroxy acids do not require deprotonation prior to peptide bond formation.[89] Even though natural amino acid polymerization by the ribosomal PTC is not pH dependent,[44] it is possible that the incorporation of β-amino acids is pH dependent. Indeed, the pK_as of β-amino acids are higher than the pK_as of natural amino acids; therefore, deprotonation could be rate-limiting step in β-amino acid incorporation. Another possibility is that compared to β-aa-tRNAs, β-OH-tRNAs may have a higher affinity for EF-Tu and the ribosomal A site. Collectively, based on the incorporation of backbone analogs reviewed

thus far, it appears that the carbon backbone analogs are incompatible with the TM, while amino acids with heteroatomic backbones are compatible with the TM.

Although significant strides were made in studying the plasticity of the ribosomal PTC for various UAA-tRNA substrates in cell-free translation extracts, there are drawbacks that limit their use. For instance, the protein yields from cell-free translation extracts were low, presumably due to competition between suppressor tRNAs and RFs. In addition, UAA mutagenesis requires stop codons, but the genetic code only has three stop codons limiting the incorporation of UAAs to two. Finally, synthesizing UAA-tRNAs using the chemical approach is difficult and time-consuming. These drawbacks served as a driving force for the development of a customizable reconstituted cell-free assay that improved the nature of the assays being implemented and introduced a new way to charge tRNAs with natural and UAAs.

Reconstituted Cell-Free Translation Systems and Sense Codon Reassignment

In 2001, the Ueda group reported the first customizable translation system, the *P*rotein Synthesis *U*sing *R*ecombinant *E*lements, PURE system.[90]

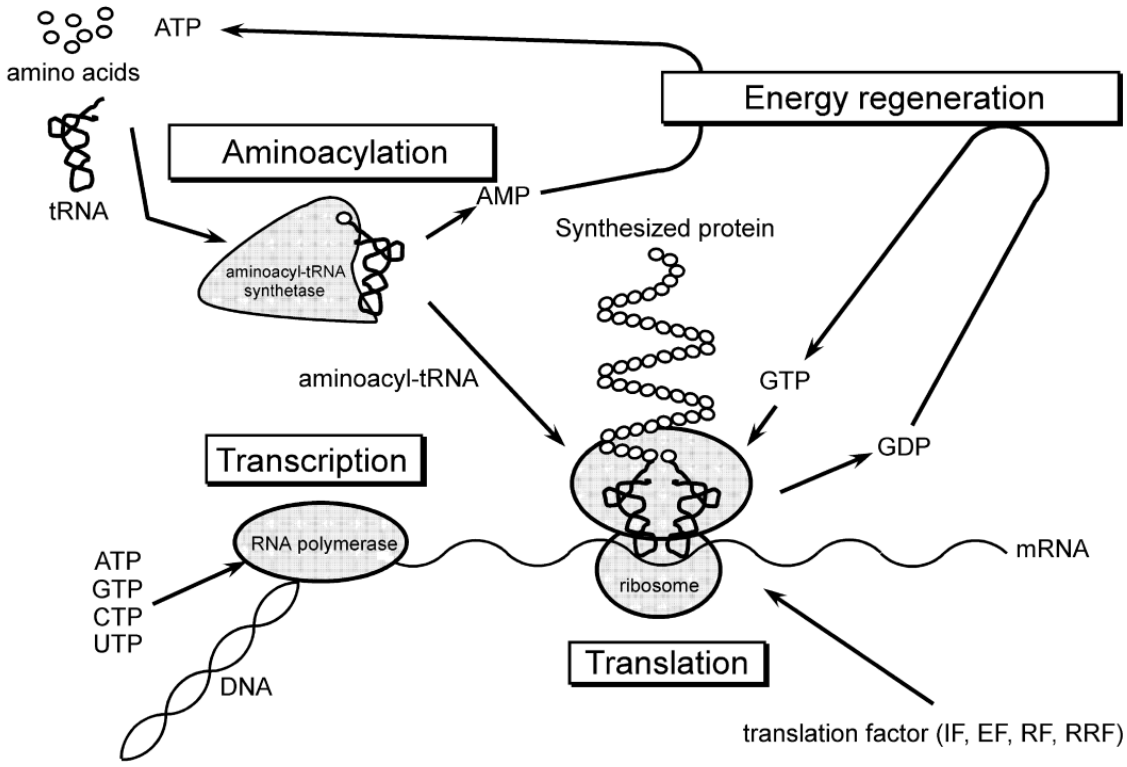

Figure 1.12 PURE Assay[90]

The PURE system consists of purifying the translation components, including all the essential translation factors (IF1, IF2, IF3, EF-Tu, EF-G and EF-Ts), tRNAs, RFs, aaRS, natural amino acids, and RRFs, from *E. coli*, and it can be used to translate five different proteins and peptides with high yields and activities. Furthermore, they were able to incorporate misacylated Val-$tRNA_{supp}$ efficiently in response to the amber stop codon by removing RF1. The PURE assay has several advantages over crude cell-free translation extracts for protein synthesis. To start, they allow complete control over all the components added to the reactions, which increases yields of both proteins containing natural and UAAs that are incorporated by stop codon suppression. Also, the PURE system does not have proteases or nucleases present, so proteins are stable, mRNAs are not degraded, and nucleotides are consumed only when translation occurs, making energy consumption efficient which results in high protein yields.[90]

The ability to exclude certain aaRS, facilitated the application of mRNA display, an approach developed to build peptide-mimetic libraries,[91] by sense codon reassignment. N-methyl amino acids, dehydroalanine, and α-amino acids with unnatural side chains were obtained by either chemically modifying

aa-tRNAs before adding them to the reaction, modifying the amino acid side chains and backbones, including cyclizing peptides, post-translationally, or generating misacylated aa-tRNAs *in situ*.[61,92–94] While the PURE system has proven to be a valuable toolkit for scientists, the fact remains that applying the PURE system for unnatural amino acid incorporation requires purifying all of the components which is time-consuming, and the presence of RFs and aaRS complicates the reassignment of multiple sense codons to UAAs. This, in turn, affects the synthesis of diverse peptide libraries.

An alternative approach to the PURE assay was developed by the Cornish lab, which involves the purification of essential translation components, such as ribosomes, mRNAs, aa-tRNAs, and initiation and elongation factors.[95] By only including the translation factors that are essential to translation, and omitting all other competing components, including aaRSs, RFs, and RRFs, all 64 sense codons can be reassigned to unnatural amino acids. This method afforded the first high-yielding mRNA directed synthesis of unnatural α-peptides possible. Figure 1.13

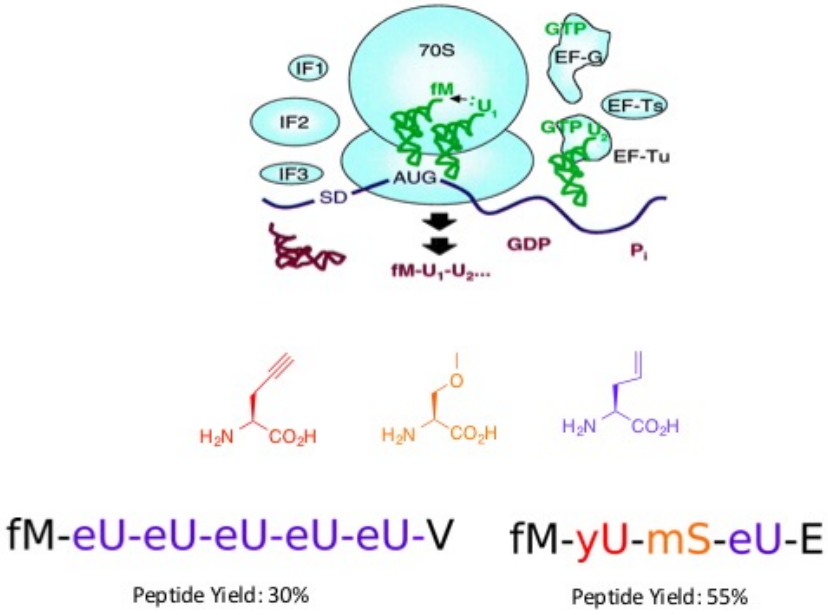

Figure 1.13 Purified Translation System[95]

Furthermore, the absence of competing factors allowed them to assay the ribosome's compatibility with unnatural backbones. They found that N-alkyl amino acids, α,α-disubstituted amino acids, and α-hydroxy acids are incorporated with high efficiencies into tripeptides, but D- and β-amino acids are not detected. Figure 1.14

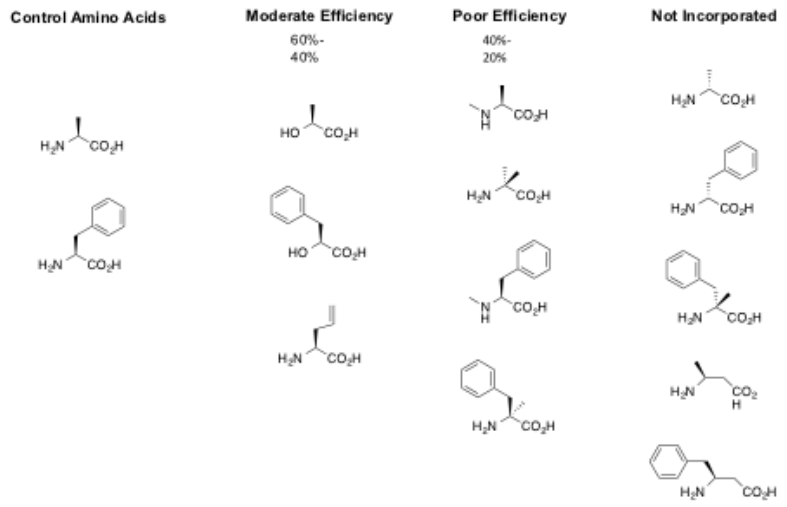

Figure 1.14 Backbone analogs incorporated with the purified translation system

Perhaps the main drawback of this particular study is that amino acid incorporation was quantified using a radioactive third amino acid, instead of the initiator tRNA being radioactive. Therefore, it was not possible to ascertain whether D- or β-amino acids were good acceptors, but poor peptidyl donors due to their inability to translocate or assume the correct spatial arrangement in the PTC for peptide bond formation following translocation. This assay was also combined with mRNA display to build peptide libraries where multiple sense codons were reassigned to α-amino acids with unnatural side chains.[96] Furthermore, it provides the perfect platform to study the mechanism of incorporation of UAAs. For example, the ribosome does polymerize linear and cyclic N-alkyl amino acids but efficiency depends on the N-substitution sterics and nucleophile basicity.[97,98] However, successful implementation of this technology requires the purification of each translation component and chemical synthesis for misacylating tRNAs, which are both time-consuming, therefore making this assay difficult to use in the large scale synthesis of unnatural backbone oligomers.

Coupling ribozyme catalyzed tRNA aminoacylation with reconstituted cell-free translation systems for the synthesis of backbone oligomers

The chemical aminoacylation of UAA-tRNAs was essentially rendered obsolete once the flexizyme was developed. The flexizyme, engineered by the Suga group, is a ribozyme that catalyzes tRNA

aminoacylation of any amino acid on any tRNA. [99,100] The flexizyme aminoacylates tRNAs by recognizing an aromatic motif on an amino acid and the conserved 3' CCA end of a tRNA.

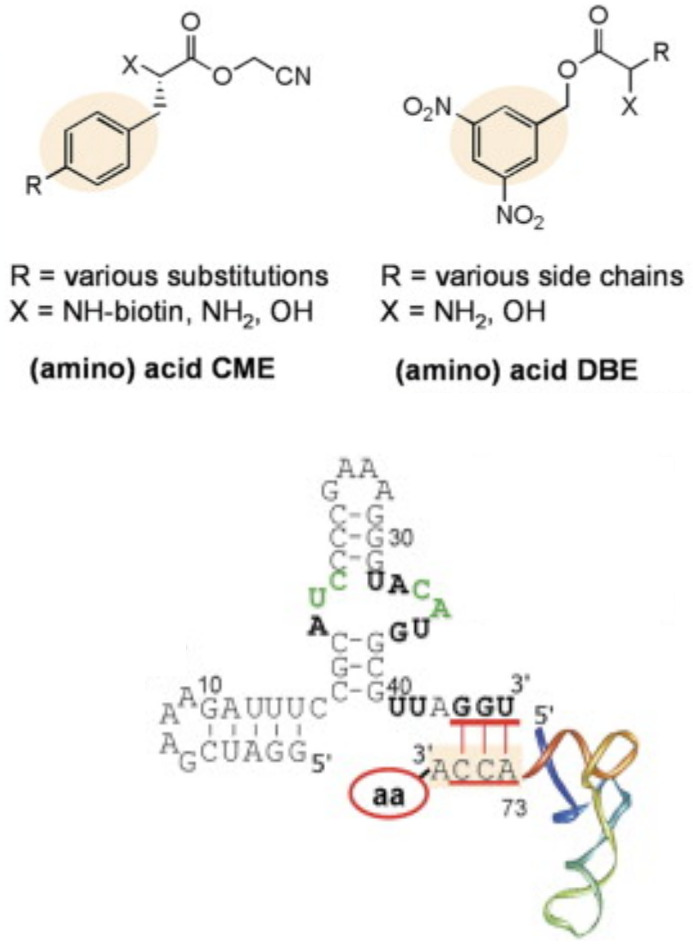

Figure 1.15 Charging tRNAs with the flexizyme[99,100]

The key to the universal aminoacylation of tRNAs using the flexizyme is that its recognition motifs are independent of the amino acid, as long as an aromatic motif is present in the amino acid side chain or on the acyl donor. Compared to the chemical aminoacylation method, charging a tRNA with the flexizyme is straightforward; there is no protein purification involved since the flexizyme is prepared using simple molecular biology techniques, such as PCR and *in vitro* run-off transcription. While the chemistry employed to prepare amino acid derivatives for tRNA charging involves multiple steps, they are straightforward and high-yielding, making this method much more accessible to scientists across many different fields. Combining this technology with a reconstituted cell-free translation system hastened the *in vitro* ribosomal

synthesis of peptides containing amino acids with unnatural side chains and backbones, including α-hydroxy acids, N-methyl amino acids, D-amino acids and β-amino acids (see below for further discussion). Furthermore, this technology is amenable to the rapid and facile engineering of monomer sequences to facilitate the post-translational cyclization of unnatural oligomers. Below, I will discuss the different amino acid backbone analogs that have been incorporated using the flexizyme aminoacylation approach.

1.1.1 α-hydroxy acids

α-hydroxy acids have been shown to be suitable substrates for the ribosome, but their incorporation had not been confirmed directly. By using chemically aminoacylating tRNAs with lactic acid and phenyl lactic acid, the TM was deemed compatible with the purified translation system.[101] The TM mediated synthesis of polyesters was achieved by using the flexizyme technology to aminoacylate tRNAs. Using this method, arbitrary codons were assigned to α-hydroxy aminoacyl-tRNAs and polyesters tagged with a C-terminal flag were synthesized.[102] This method was also used to achieve the mRNA-directed polyester synthesis with specific sequences.[103] In both cases, the resulting polyesters contained a C-terminal flag peptide, so when they were base treated, the resulting α-hydroxy-flag peptides were analyzed using MALDI-TOF and gel electrophoresis. Based on the product's molecular weight and migration by gel electrophoresis, it was shown that the TM can synthesize up to 12-mer polyesters. However, in both cases, they were unable to incorporate more than 12 α-hydroxy acids, probably due to the slow ribosomal formation of polyesters and the low affinity of α-hydroxy acids for EF-Tu,[102] thus limiting the number of α-hydroxy aminoacyl-tRNAs that can be incorporated *in vitro*.

1.1.2 N-alkyl amino acids

N-alkyl amino acids have been one of the more amenable backbone analogs to be incorporated by the ribosome in cell-free translation systems, and similar results were obtained in the purified translation systems. Sense codon suppression has shed light on the single site and multiple site incorporation of N-alkyl amino acids into linear as well as cyclic N-alkyl peptides.[104,105] For example, N-alkyl amino acids are incorporated into peptides as long as the N-substitutions are non-bulky, aliphatic or aromatic; bulky and

charged N-substituted amino acids are not incorporated. As long as these requirements are followed, ribosomes can synthesize oligomers containing up to four consecutive N-alkyl amino acids.

1.1.3 D-amino acids

D-aminoacyl tRNAs were shown to be successful initiator tRNAs.[106] Furthermore, the incorporation of D-amino acids with yields ranging from 10%-65% was achieved by optimizing the EF-Tu and aa-tRNAs concentrations to facilitate D-aa-tRNA incorporation.[107] Combining the flexizyme technology with the purified, reconstituted translation system allowed for the first mechanistic study of D-aa incorporation to investigate why the efficiency of D-amino acid incorporation has been reported as low. [108] D-aa-tRNAs were incorporated into di- and tripeptides to test their donor and acceptor abilities. They showed that D-amino acids are efficiently delivered to the ribosomal A site, they are efficient peptide chain acceptors, and the resulting peptidyl-tRNA containing the D-aa is translocated efficiently. However, the D-amino acid containing peptidyl-tRNA are poor peptide chain donors. The inability of D-amino acid containing peptidyl-tRNA to act as efficient peptide chain donors leads to a large population of ribosomes that are arrested, and a small population that continues to translate efficiently. The translational arrest of the ribosomes was attributed to the D-amino acid rendering the PTC inactive.

1.1.4 β-Amino Acids

In 2016, the Suga group published the first report of multiple β-amino acid incorporations by a purified translation system.[109] Just like with D-amino acids, their approach consisted of optimizing their reconstituted translation system by adjusting the EF-Tu and β-aa-tRNA concentrations to facilitate ternary complex formation, β-aminoacyl-tRNA delivery to the ribosome, and β-aminoacyl-tRNA accommodation on the A site. Using an mRNA coding for five natural amino acids, followed by the β-amino acid and a FLAG peptide, they found they were able to incorporate 13 different β-amino acyl-tRNAs with 50% efficiency. They noted that as the size of the β-aminoacyl-tRNA side chain increased, the lower the β-amino acid incorporation yield obtained. When they tried to translate two consecutive β-aa-tRNAs, they did not obtain the expected peptide. However, when they added a natural amino acid spacer between two β-amino acids, the yields were recovered. They attributed this result to the conformational flexibility two β-aminoacyl-tRNAs

in the PTC might prevent these aa-tRNAs to adopt the proper conformation for peptide bond formation. This result led them to incorporate three β-aminoacyl-tRNAs with a natural amino acid spacer that varied between one, two and, three amino acids. They credited the success of this result to the fact that multiple β-aa-tRNA incorporations might require an amino acid linker between each β-amino acid.

Evolving the TM to incorporate D- and β-amino acids

One way to circumvent the intractability of D- and β-amino acids with the TM is to mutate the PTC bases that are implicated in peptide bond formation. This approach was first tested with D-amino acids. The PTC was mutated using directed evolution to yield mutant ribosomes that accommodate the D-amino acid stereochemistry, and as a result, incorporate D-amino acids.[110,111] These results motivated the Hecht group to mutate the PTC to yield mutant ribosomes that incorporate β-amino acids.[112–114] Based on the observation that β-puromycin inhibit translation even though they have low affinity for eukaryotic ribosomes,[115] the Hecht group screened a library of ribosome with PTC mutations. The PTC mutations used in this report were adapted from a previous report where mutant ribosomes that are not as sensitive to D-amino acids were discovered.[110] To select ribosomes with the desired mutations, they used a double selection with β-puromycin and erythromycin, an antibiotic that inhibits PTC activity. β-puromycin allowed them to select for ribosomes that could tolerate the β-aminoacyl moiety, while erythromycin allowed them to select against wild-type ribosomes *in vivo*. Based on their double selection, the Hecht group obtained S-30 extracts with mutant ribosomes that not only incorporate natural amino acids but also β-alanyl-$tRNA_{CUA}$ in response to the UAG stop codon in DHFR, *P. pyralis* firefly luciferase, and *O. madagascariensis* scorpion IsCT peptide.[113] These mutant ribosomes were later tested for their ability to incorporate β-Phe β,β-dimethyl-β-alanine, β-(p-bromophenyl)alanine, and α-methyl-β-alanine in response to the UAG stop codon embedded in the middle of the DHFR sequence using S-30 extracts.[112] In order to incorporate different stereo- and regioisomers of β-amino acids, the Hecht group subjected the library of ribosome mutants to two other rounds of directed evolution using two additional β-puromycin analogs, where the R group is attached to the C2 (β^2-puromycin) and C3 (β^3-puromycin).[114] Their search resulted in mutant ribosomes that could incorporate β^2-alanine and β^3-alanine with R and S configurations at the C2 and C3 carbon respectively into full-length DHFR. Figure# While these mutant ribosomes provide an alternative for the

incorporation of β-amino acids into full proteins, and they have been used to incorporate β^3-Phe-$tRNA^{Phe}$ analogs *in vivo*,[116] both methods has several limitations. The *in vitro* translations are carried out in the presence of RFs that compete with stop codon suppression. The *in vivo* incorporations are limited by the number of sense codons can be replaced, which would limit the synthesis of diverse β-peptide libraries.

Summary and Thesis Overview

The TM is an important biosynthetic tool for building large libraries of synthetic peptides. However, the TM machinery is limited by the types of monomers it accepts, which limits the diversity of peptide-mimetic libraries. While TM structural and mechanistic studies has expedited the development of technologies to synthesize peptides with unnatural backbones, such as α-hydroxy acids and N-alkyl amino acids, D- and β-amino acids continue to be difficult substrates for the natural TM. Because modifying the TM is labor-intensive, identifying alternate ways of incorporating these intractable monomers remains an important goal.

In this thesis, I use β-amino acid backbone to yield α-hydroxy-β-amino acids. The α-hydroxy moiety provides an alternate pathway for the incorporation of β-amino acids using the natural TM. In **Chapter 2**, I describe the proof-of-concept experiments that show that α-hydroxy-β-aminoacyl-tRNAs are suitable A-site and P-site substrates. I show that these monomers are incorporated through the formation of an ester bond that undergoes a rapid rearrangement to yield an amide bond. In **Chapter 3**, I expand on the single incorporation of α-hydroxy-β-amino acids results to show that the ribosome incorporates multiple α-hydroxy-β-amino acids and discuss the limitations of this technology. Finally, I list the materials and methods that were performed in **Chapter 4**.

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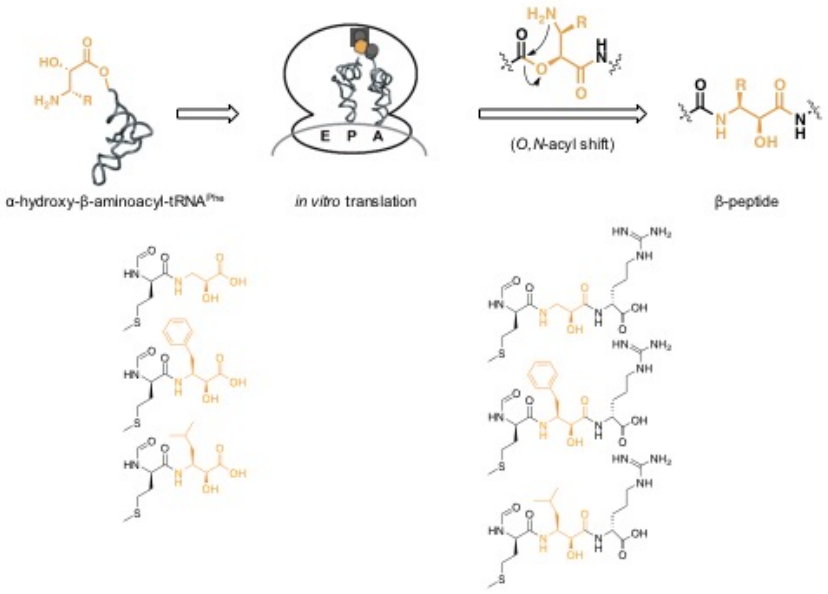
2 Chapter 2 The ribosomal incorporation of single α-hydroxy-β-amino acids

Introduction

β-amino acids are important building blocks for the synthesis of biologically relevant peptidomimetics because they provide enhanced proteolytic stability *in vivo and in vitro*.[1] However, the full therapeutic potential of β-amino acid-based peptidomimetics has not been realized due to their limited production by time-consuming synthetic methods, such as solid phase peptide synthesis (SPPS). While SPPS approaches are applicable to any building block, a small peptide on the order of 41 amino acids can take up to 82 hours to synthesize, which is not practical for the large-scale production of peptidomimics.[2] In addition, SPPS becomes increasingly inefficient as the size of the peptide grows, due to incomplete deprotection and coupling steps.[3,4]

Thus, researchers have turned to other methods to ease the production of β-amino acid containing proteins and peptides. One alternative approach involves the expressed protein ligation method, which involves attaching a synthetic peptide to a recombinant protein.[5] Although this method can successfully produce proteins containing unnatural amino acids, the modified protein requires several steps to assemble, including the overexpression and purification of the recombinant protein, the synthesis of the unnatural peptide, and the actual ligation step. Another approach uses the translation machinery to incorporate β-amino acids. Initially, β-amino acids were considered intractable ribosomal substrates, however, the Suga group was able to synthesize β-amino acid-based peptides *in vitro* by adjusting the EF-Tu and aa-tRNA concentrations.[6] Although Suga and coworkers were able to successfully adapt the translation machinery to produce β-amino acid peptides, they reported only 50% sense suppression relative to the control peptide.

Here, we propose a method that improves the adaptation of the translation machinery (TM) for the production of β-amino acid peptides by modifying the β-amino acid carbon backbone to increase its incorporation *in vitro*. Because the ribosome can polymerize α-hydroxy acids,[7–13] and acyl shifts are well documented in the literature,[14–16] we hypothesized that the presence of the α-hydroxy moiety would encourage the ribosome to incorporate α-hydroxy-β-amino acids via the formation of an ester bond that would rapidly rearrange to form the amide bond (Scheme 2.1).



Scheme 2.1 Proposed mechanism for the ribosomal incorporation of α-hydroxy-β-amino acids

In this work, I test the α-hydroxy-β-amino acid rearrangement hypothesis by measuring the conversion of ^{35}S-fMet-tRNA^{fMet} to di- and tripeptides using a reconstituted *in vitro* translation system with three α-hydroxy-β-aminoacyl-tRNAs. I successfully demonstrate that the ribosome incorporates α-hydroxy-β-aminoacyl-tRNAs through the formation of an ester bond that is uniquely positioned to undergo an O, N acyl shift to yield a native peptide bond. Moreover, α-hydroxy-β-aminoacyl-tRNAs are incorporated with efficiencies comparable those observed with natural substrates. This approach represents the first time the functionalization of an unnatural amino acid backbone results in the successful ribosomal incorporation of a previously intractable substrate.

Results

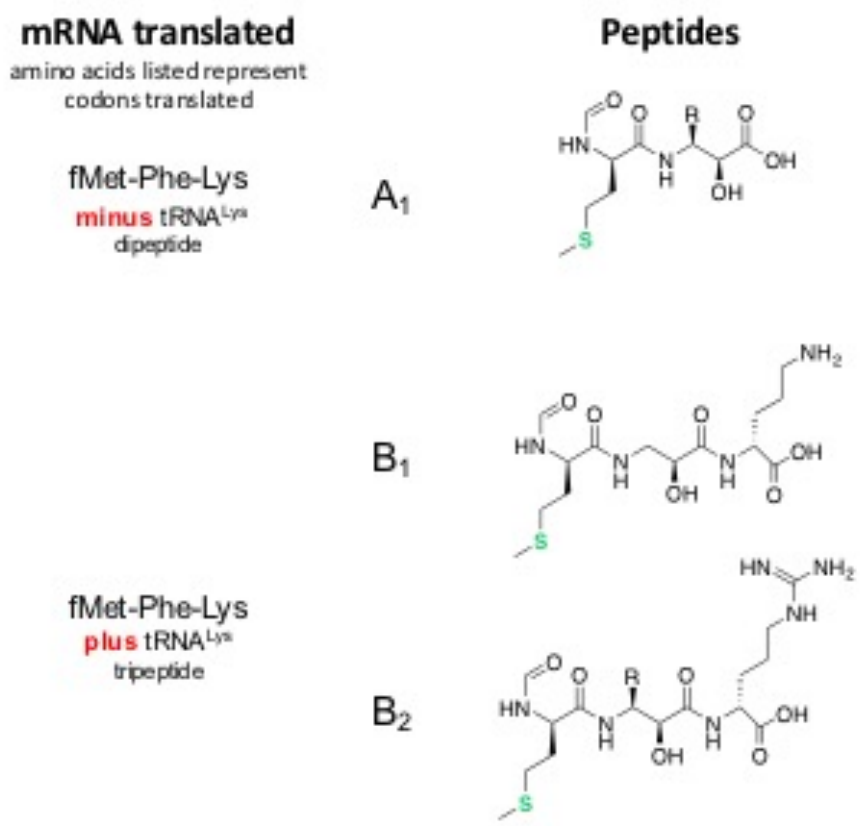
2.1.1 Selection and Synthesis of α -hydroxy-β-aminoacyl-tRNAs

To probe the ribosomal compatibility of α-hydroxy-β-amino acids, we selected isoserine (Iso, **B**), (2S,3S)-3-amino-2-hydroxy-4-phenylbutyric acids (β-Phe, **C**), and (2*S*,3*S*)-3-amino-2-hydroxy-5-methylhexanoic acid (β-Leu, **D**), which both vary in size, hydrophobicity, and electrostatics. The α-hydroxy-β-amino acids were charged onto tRNA^{Phe} to ensure that peptide bond formation was not inhibited by high

tRNA body binding affinity to the ternary complex.[17] Using the flexizyme technology, the $tRNA^{Phe}$ was misacylated according to the procedure outlined by Suga et.al.[18]

2.1.2 Framework for evaluating the ribosomal compatibility of α -hydroxy-β-aminoacyl-tRNAs

The reconstituted translation system was as previously described.[19] Based on this system, two assays were designed to study the incorporation of the different α-hydroxy-β-aminoacyl-tRNAs - a dipeptide assay and a tripeptide assay. The dipeptide assay evaluates the compatibility of α-hydroxy-β-aminoacyl-tRNAs with the translation machinery and its acceptor activity. The tripeptide assay evaluates α-hydroxy-β-aminoacyl-tRNAs peptide bond donor activity. Because translations are initiated [^{35}S]-fMet-$tRNA^{fMet}$, peptide products were quantified by electrophoretic thin layer chromatography (eTLC). Since eTLC resolves peptides based on their charge, Lys-$tRNA^{Lys}$ or Arg-$tRNA^{Lys}$ were selected as the third amino acid. To confirm the identity and yields of the peptides, I mixed and co-injected the radioactive translation product with the chemically synthesized authentic peptide marker, and analyzed them on an HPLC with a β-RAM and a UV detector. The β-RAM detector corroborated radioactive peptide yields obtained by eTLC, while co-migration of the β-RAM signal and UV signal validated the peptide identity. The expected peptide products are shown in Scheme 2.2.



Scheme 2.2 Expected translation products with α-hydroxy-β-amino acids

2.1.3 Ribosomal synthesis of dipeptides containing a single α -hydroxy-β-amino acid

Using α-hydroxy-β-aminoacyl $tRNA^{Phe}$ with our purified *in vitro* translation system, and analyzing the peptide products by eTLC and HPLC β-RAM, I demonstrate that the ribosome synthesizes dipeptides containing α-hydroxy-β-amino acids (Figure 2.1). I observed ≥70% radioactive fMet converted to both natural and unnatural dipeptide, suggesting that the yields obtained with α-hydroxy-β-aminoacyl-$tRNA^{Phe}$s are comparable to what was obtained with the control Phe-$tRNA^{Phe}$. Finally, my dipeptide results indicate that α-hydroxy-β-aminoacyl $tRNA^{Phe}$ are efficient peptide donors.

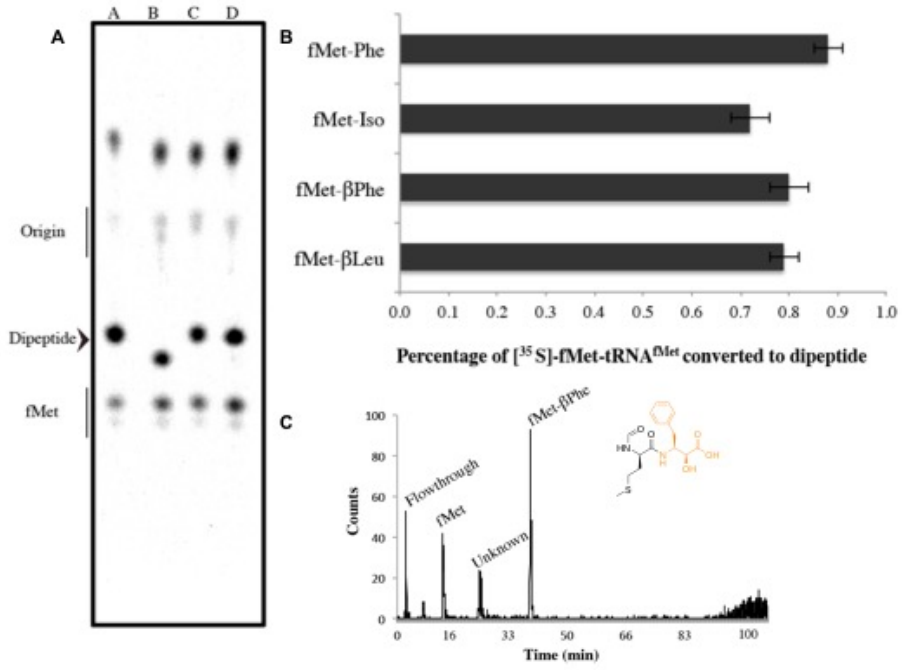


Figure 2.1 Ribosomal incorporation of α-hydroxy-β-amino acids into dipeptides

2.1.4 Ribosomal synthesis of tripeptides containing a single α-hydroxy-β-amino acid

To determine the ribosome's ability to act as peptide acceptors, and therefore their potential for any future application involving the ribosomal polymerization of α-hydroxy-β-amino acid, I translated the tripeptide B_1. Even though the yields for the tripeptide were somewhat lower than for the dipeptides (50-60% fMet conversion to tripeptide), we were able to show by eTLC that α-hydroxy-β-aminoacyl-$tRNA^{Phe}$ is also an efficient peptide acceptor during translation. Curiously, when we attempted to confirm these results using HPLC-β-RAM co-injection with the synthetic authentic markers, the authentic marker trace and translation trace peak did not co-migrate. These results suggested that while Lys was incorporated, the identity of the final peptide did not match the authentic peptides. Based on these results, I hypothesized that the lysine side chain was interfering with the expected O, N acyl shift. To address this problem and minimize the number of changes to the system, I charged Arg onto $tRNA^{Lys}$ using the flexizyme technology discussed in Chapter 1, and translated peptide B_2. When Lys was replaced with Arg, the translation yields were not only higher by eTLC (≥70%), the authentic markers matched the authentic tripeptide peak and the yields observed by eTLC (Figure 2.2).

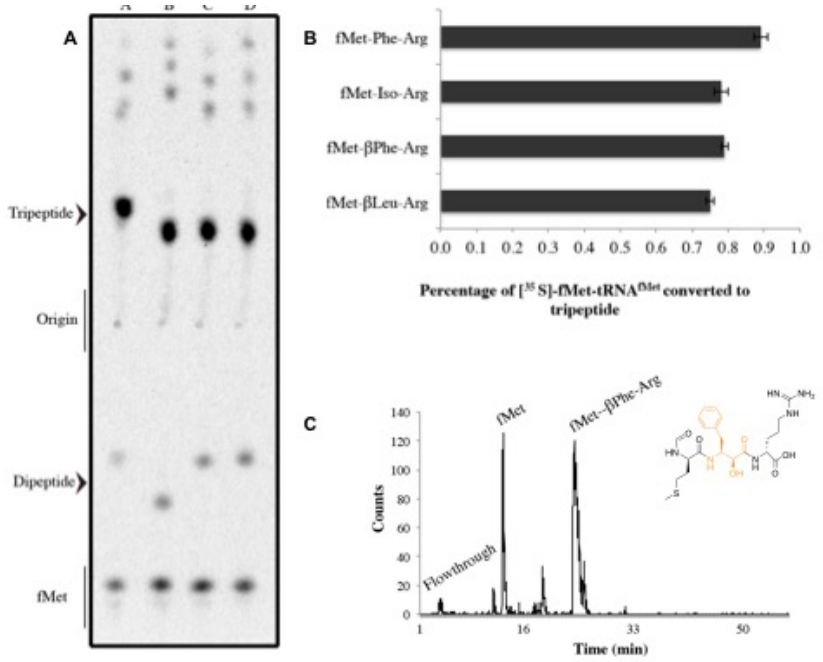
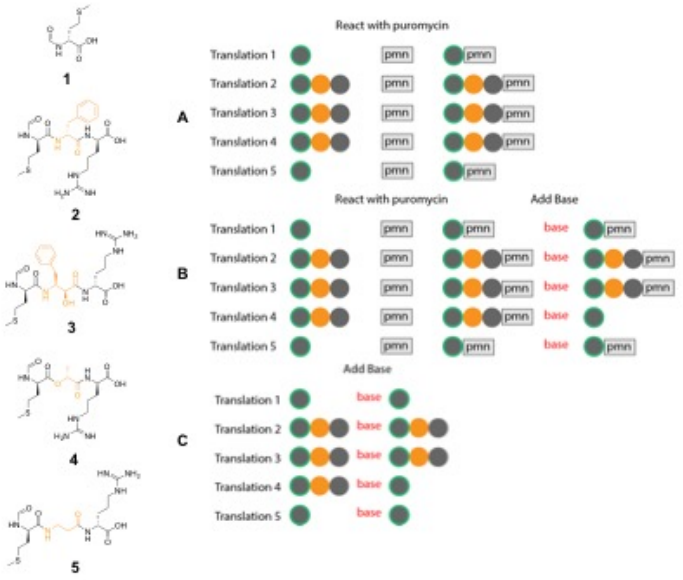


Figure 2.2 Ribosomal incorporation of α-hydroxy-β-amino acids into tripeptide

2.1.5 Control Experiments

Next, we tested our hypothesis for the ribosomal incorporation of α-hydroxy-β-aminoacyl-tRNA^{Phe} detailed in Scheme 1. Based on the ribosome's ability to incorporate esters, we postulated a two-step mechanism for the incorporation of α-hydroxy-β-amino acids. First, α-hydroxy-β-aminoacyl tRNA^{Phe} would form an intermediate ester bond. Because the amine is unique positioned to attack the ester's carbonyl carbon, the peptide would undergo a spontaneous O, N acyl shift to yield a base-stable amide bond. The feasibility of this premise was tested by replacing isoserine-tRNA^{Phe} with two control aa-tRNAs, Lac-tRNA^{Phe} and β-alanine-tRNA^{Phe}. These two control aa-tRNAs were chosen as they are Iso analogs. Lac-tRNA^{Phe} has the α-hydroxy moiety but lacks the α-amine, while β-alanine-tRNA^{Phe} contains the α-amine but lacks the α-hydroxy moiety. The experimental conditions and expected results are detailed in Scheme 2.3 Cartoon representation of control reactions.



Scheme 2.3 Cartoon representation of control reactions

To determine whether the α-hydroxy-β-amino acids are incorporated via an ester bond that rearranges to form an amide bond, I quenched the four tripeptides, 2, 3, 4, and 5 with the three conditions detailed in Scheme 3 and compared the resulting eTLC patterns. First, I incubated the translation reactions with puromycin (pmn) (Scheme 2.3) because it releases the peptide chain from the peptidyl tRNA without interfering the ester backbones. Then, I treated the puromycin-terminated peptides with base (Scheme 3B). As a result, any peptides with an ester bond would be cleaved and result in an increase of fMet observed by eTLC. Finally, treating the peptides only with base would cleave all the ester bonds in the reaction (Scheme 2.3). Most importantly, if the amino acid is not being incorporated into the peptide chain, we would observe fMet-pmn with the first two conditions and fMet with the third condition.

The results of the control experiments are shown in Figure 2.3 Control reactions. The control peptide 2 contains an amide bond, and therefore the patterns observed by eTLC are representative of peptides with base-stable amide backbones. The patterns observed with the control peptide 4 correspond to the presence of an ester bond in the peptide backbone. When peptides with ester backbones are released with puromycin (Figure 2.3, lane 4a), we observe the formation of a tripeptide. However, when peptide 4 is released with puromycin and then base treated (Figure 2.3, lane 4b) or just base treated,

(Figure 2.3, lane 4c), only fMet is observed. Control peptide 5 contains a classic, unaltered β-amino acid that is not compatible with the TM.[12] The patterns observed with peptide 5 illustrate is consistent with the unsuccessful incorporation of β-aminoacyl-tRNAs by the TM. When I evaluate the patterns observed with peptide 3 with the three conditions outlined above, it is clear that the resulting peptide contains an amide bond, and not a base-labile ester bond (Figure 2.3, Lanes 3a, 3b, 3c). This result validates my conclusion that the ribosome incorporates α-hydroxy-β-aa-tRNAs through the formation of an ester bond that rapidly rearranges through an O, N acyl shift to yield the native peptide bond.

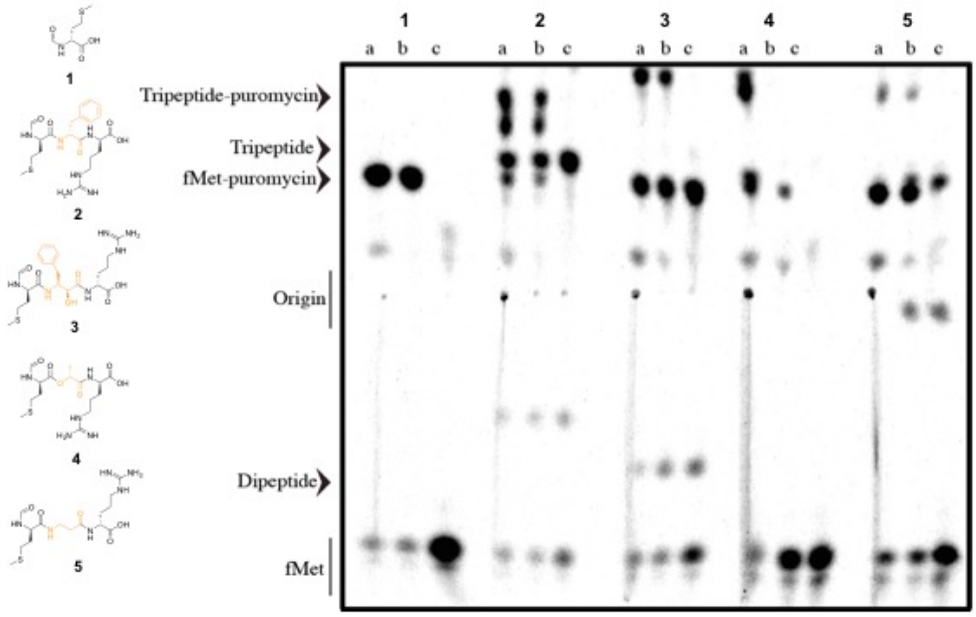


Figure 2.3 Control reactions

Note: Lane 1 is the control lane to visualize where fMet and fMet-pmn migrates by eTLC. Lane 3c indicates the peptide 3 and fMet-pmn co-migrate by eTLC

2.1.6 Discussion

Peptidomimetics have many potential therapeutic applications, as they assume secondary structures akin to the secondary structures observed in natural proteins, and therefore act as efficient modulators of protein activities.[20] However, their synthesis, which mostly relies on SPPS, limits the length of the peptides and restricts the size and diversity peptidomimetic libraries. Therefore, using the TM would provide an alternative way to synthesize these important targets. Unfortunately, most reports suggest that

the natural TM discriminates against amino acids with unnatural backbones, such as β-amino acids.[10,12,21,22] In this study, we proposed altering the β-amino acid backbone by functionalizing the α-carbon with a hydroxyl group, which would ideally to lead the ribosome into incorporating β-amino acids through the formation of an ester bond that rapidly rearranges to form an amide bond.

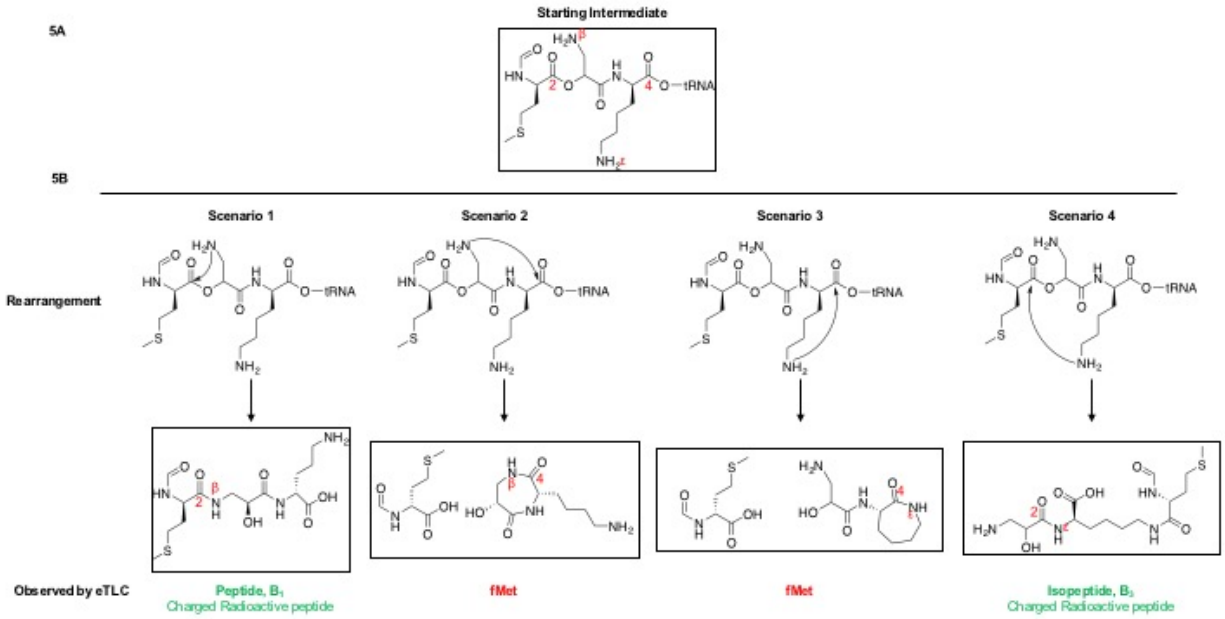
My results indicate that the O, N acyl rearrangement is compatible with the TM and is independent of side chain structure. Furthermore, base quenching resulted in di- and tripeptides, and not fMet, confirming that the O, N acyl shift is complete after 1h. This conclusion is supported by our control experiments with Lactic acid and β-alanine, which confirm that α-hydroxy acids are compatible with the translation machinery, but β-amino acids are not. Therefore, if α-hydroxy-β-aminoacyl-tRNA^{Phe}s were being incorporated via the primary amine, I would most likely observe low peptide yields or no peptide formation. However, because I observe yields that are comparable to those obtained with the control peptide, the mechanism is validated.

The eTLC patterns were identical when Lys (Scheme 2.2, B_1) or Arg (Scheme 2.2, B_2) were in the third position, but the B_2 yields are about 20% higher than B_1 yields. Furthermore, when B_1 translations were co-injected with the synthetic authentic markers, the β-RAM traces did not align with the UV trace from the chemically synthesized authentic marker. However, when I co-injected the B_2 translations with the corresponding authentic marker, I confirmed that B_2 connectivity is consistent with the expected natural amino acid connectivity.

My results indicate that Lys interferes with the formation of expected peptide, B_1. By keeping the tRNA^{Lys} body consistent and changing the identity of the charged amino acid from Lys to Arg, I was able to rule out any problem with the tRNA body. I concluded that the lysine side chain is interfering with the rearrangement chemistry. Below, I discuss a mechanistic model that might explain the results obtained with Lys-tRNA^{Lys}.

2.1.7 A proposed mechanistic model for the formation of B_3

I envision three distinct scenarios where the Nε could be interfering with the O,N acyl shift by reacting with one of the two electrophilic carbons, labeled C_2 and C_4 in Scheme 5A.[1] These four scenarios are outlined in Scheme 2.4.



Scheme 2.4 Possible rearrangement mechanisms for the formation of isopeptide

Scenario 1: N_β attacks C_2, a 5-membered ring intermediate forms during O,N acyl shift, yielding a translation product, whose β-RAM trace aligns with the UV authentic marker trace.

Scenario 2: N_β attacks C_4, forming a 7-membered ring intermediate before the O,N acyl occurs. Upon quenching with base, the ester bond that links the 7-membered ring to fMet is cleaved and only fMet would be visible by eTLC.

Scenario 3: Nε attacks C_4 to form a 7-membered ring lactam that is bonded to fMet by an ester bond. When the lactam is treated with base, fMet would be the only visible product.

Scenario 4: If N_β attacked C_4, then an 11-membered ring intermediate would form during the O,N acyl shift. This leads to the formation of a base-stable isopeptide, B_3.

[1] This mechanism does not include the amides, which could also act as nucleophiles in the presence of water when subjected to extreme heat.[30]

My results seem to be consistent with Scenario 4. In view of the fact that the ribosome's catalytic activity is entropy driven,[23] I can infer that the lysine side chain is uniquely positioned to react with the ester intermediate before the expected O, N acyl shift occurs. This stabilization would yield isopeptide, B_3. Since eTLC detects the charge of the peptides and not their connectivities, B_3 is resolvable by eTLC but its altered connectivity affects HPLC retention times. Therefore, the β-RAM traces do not match the authentic marker traces. While the ribosomal synthesis of B_3 would rely on the 11-membered ring formation, which is rarely observed probably due to unfavorable steric interactions of 11-membered rings, there is a recent report that suggests 11-membered ring intermediates are possible with S, N acyl shifts.[24] Furthermore, because the pre- and post-catalysis X-ray structures published by the Ramakrishnan laboratory that suggest that the peptidyl transferase center (PTC) is dynamic, we could suggest that the PTC might accommodate and stabilize the 11-membered ring transition state.[25]

Conclusion and Future Directions

The single site incorporation of α-hydroxy-β-amino acids using our reconstituted *in vitro* translation system provides a robust approach for synthesizing peptides and proteins containing β-amino acids monomers. Intramolecular and intermolecular acyl shifts have been used in native chemical ligation to create longer proteins,[26,27] synthesize small molecule drugs,[28] and observed in autoproteolysis.[29] However, to our knowledge, this is the first time that an intramolecular O,N acyl shift has been applied in the ribosomal synthesis of peptides containing unnatural backbones. Even more striking, this provides a novel approach for the synthesis of isopeptides by the ribosome. While the identity of the isopeptide is yet to be directly confirmed by co-migration with the authentic isopeptide marker, both of these approaches are widely useful for the synthesis of backbone analogs of natural peptides. For example, if we replace the α-hydroxy with an α-thiol, the peptide could be incorporated through the formation of a thioester that would undergo a spontaneous S,N acyl shift to yield peptides with the α-thiol-β-amino acid backbone. Subjecting these peptides to reducing conditions using Raney Nickel would yield a peptide with an unsubstituted β-backbone analog. Another interesting study would involve the polymerization of α-hydroxy-β-amino acids to yield peptides with multiple α-hydroxy-β-amino acids in their backbone, which I explore in Chapter 3.

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3 Chapter 3 The ribosomal incorporation of multiple α-hydroxy-β-amino acids

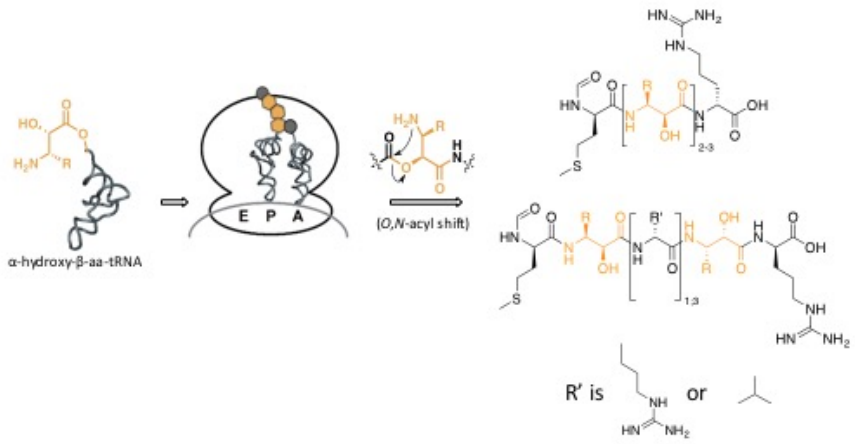
Introduction

β-peptides foldamers are peptidomimetics that mimic native protein folding patterns under physiological conditions.[1] Unlike their α-peptide analogs, β-peptides, and specifically α-hydroxy-β-amino acids, are inherently stable to protease degradation.[2,3] Notably, β-peptides foldamers have been engineered to target several aberrant protein protein interactions (PPIs) such as the p53•hDM2 interaction, which has been implicated in tumor growth, and gp41, which is required for HIV host cell infection.[4–6] Despite their promise, the main strategy used to synthesize β-peptides foldamers has relied on solid phase peptide synthesis (SPPS), which is not optimal for the synthesis of diverse peptide libraries.[7] Therefore, developing new techniques to quickly and efficiently synthesize β-peptides foldamers and to generate structurally diverse libraries for high-throughput screening will facilitate the discovery of new drugs to treat diseases associated with aberrant PPIs.

The reconstituted *in vitro* translation system has been manipulated to synthesize peptides with a variety of unnatural amino acids (UAAs). In this system, UAAs are incorporated into a growing peptide chain by reassigning sense codons in the messenger RNA (mRNA) to specific UAAs by removing any competing factors, such as RF and aaRS.[8] Most importantly, reconstituted translation systems can be combined with *in vitro* selection technologies, such as mRNA display, to identify unnatural peptides that bind to specific protein targets.[9] However, using display technologies to synthesize β-peptides foldamers requires that β-aminoacyl-tRNAs are compatible with the translation machinery (TM). Unfortunately, several reports suggest that β-aminoacyl-tRNAs are incompatible with the natural TM.[8,10,11]

In Chapter 2, I showed that α-hydroxy-β-amino acids are efficiently incorporated by the TM. Analysis of di- and tripeptide translations by electrophoretic thin layer chromatography (eTLC) and HPLC β-RAM revealed that α-hydroxy-β-amino acids function as peptide chain acceptors and peptide chain donors. In addition, I showed that these new substrates are incorporated as esters that undergo a rapid O, N acyl shift to yield a native peptide bond. Given these initial successes, I next set out to test the ability of the ribosome to incorporate α-hydroxy-β-amino acids at multiple sites within the same peptide. I hypothesized that multiple α-hydroxy-β-amino acids can be incorporated through the formation of a polyester, that would subsequently undergo consecutive O, N acyl shifts to yield a polyamine. Since the

ribosome-catalyzed polyester synthesis has been validated on three different occasions,[13–15] I hypothesized that we could use the reconstituted *in vitro* translation system to synthesize peptides with multiple consecutive α-hydroxy-β-amino acids as well as peptides with alternating α-amino acids (Scheme 3.1).



Scheme 3.1 Multiple site incorporation of α-hydroxy-β-amino acids

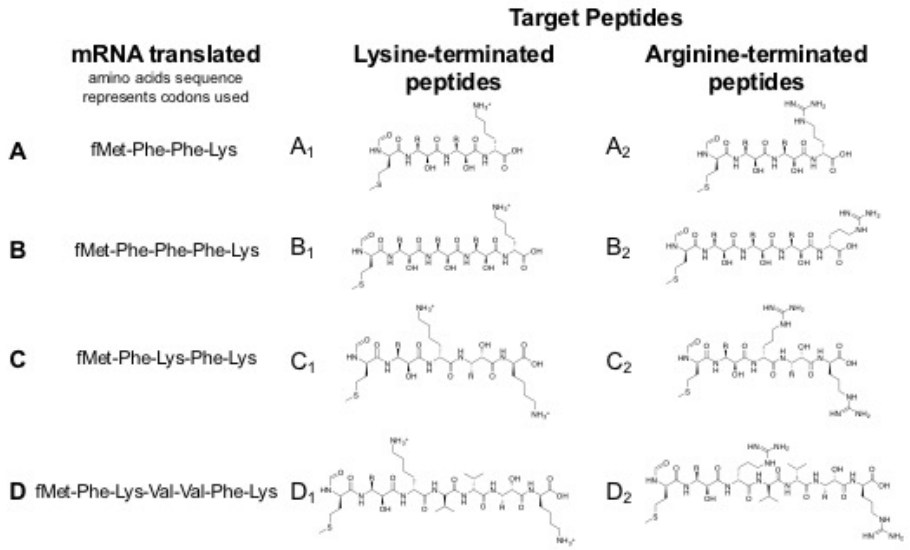
If successful, this would provide a new avenue for synthesizing libraries of structurally diverse peptides containing α-hydroxy-β-amino acids *in vitro*, and would therefore have important therapeutic implications.[16,17]

Results

3.1.1 Developing a framework to evaluate the ribosomal incorporation of multiple α-hydroxy-β-amino acids

To investigate the effect of side chain size, hydrophobicity, and electrostatics on the ribosomal incorporation of multiple α-hydroxy-β-amino acids, $tRNA^{Phe}$ was charged with its cognate amino acid, phenylalanine (Phe; a), and with three distinct α-hydroxy-β-amino acids: Isoserine (Iso, b), (2S, 3S)-3-amino-2-hydroxy-4-phenylbutyric acid (β-Phe, c), and (2*S*,3*S*)-3-amino-2-hydroxy-5-methylhexanoic acid (β-Leu, d).

Four different mRNAs encoding the peptides indicated in Scheme 3.2 were used in translation experiments. These include mRNAs encoding two consecutive α-hydroxy-β-amino acids (Scheme 3.2; mRNA A; target peptides A_1^{b-d} and A_2^{b-d}), three consecutive α-hydroxy-β-amino acids (Scheme 3.2;mRNA B; target peptides B_1^{b-d} and B_2^{b-d}), two α-hydroxy-β-amino acids separated by a single α-amino acid spacer (Scheme 3.2; mRNA C; target peptides C_1^{b-d} and C_2^{b-d}), and two α-hydroxy-β-amino acids separated by a three α-amino acid spacer (Scheme 3.2; mRNA D; target peptides D_1^{b-d} and D_2^{b-d}). Note that because eTLC resolves peptides based on their charge, I chose Lys (A1, B1, C1 and D1) or Arg (A2, B2, C2, and D2) as the final amino acid to facilitate separation and quantification by eTLC.



Scheme 3.2 Experimental setup for the ribosomal incorporation multiple of α-hydroxy-β-amino acids

3.1.2 The ribosome can incorporate two consecutive α-hydroxy-β-amino acids - Target Peptides A_1 and A_2

To investigate the efficiency with which the ribosome can incorporate two consecutive α-hydroxy-β-amino acids, I began by translating the mRNAs encoding tetrapeptide A_1^{a-d} and A_2^{a-d} . The tetrapeptide yields for A_1^{a} and A_2^{a}, the positive controls containing two consecutive Phe residues, were in both cases ~80% (Figure 3.1). Relative to this control reaction, the yields for the tetrapeptides containing two consecutive α-hydroxy-β-amino acids were ~3 to 4-fold lower (20-25%) when the peptides ended with Lys, but only ~1.5 to 2-fold lower (40-55%) when the same peptides ended in Arg. These results are consistent with the results described in Chapter 2 where I showed that tripeptide yields for a single β-amino acid

incorporation event were greater when the peptide ended with Arg. Taken together, these results suggest that the ability of β-amino acids to function as peptide-chain donors is controlled at least in part by the nature of the peptide-chain acceptor in the A site.

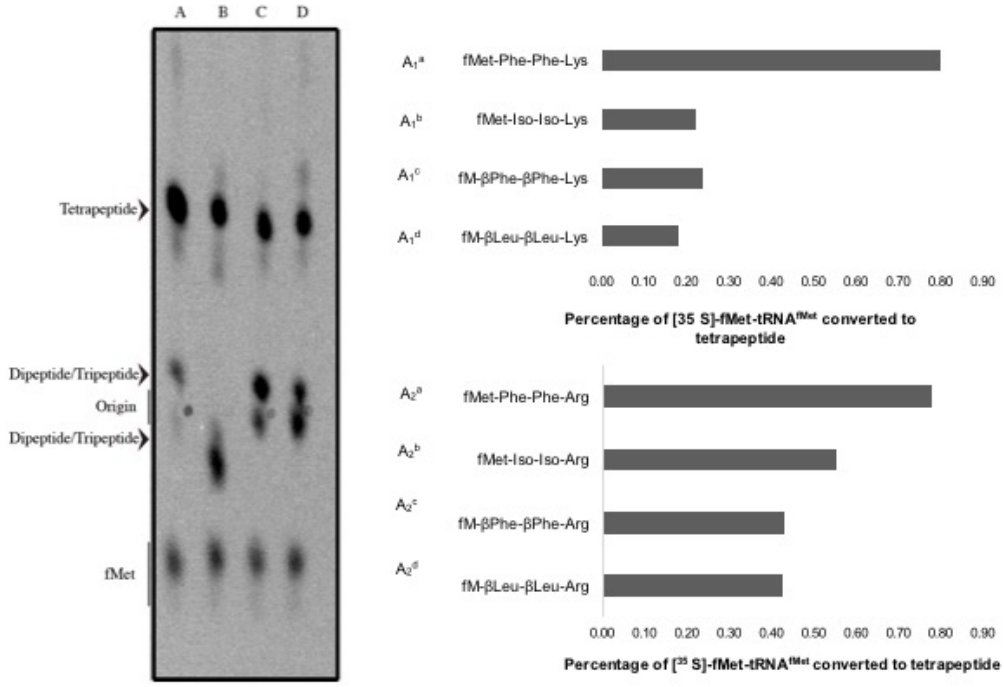


Figure 3.1 The ribosomal incorporation of two, consecutive α-hydroxy-β-amino acids

Note: The eTLC to the left represents the translations A_2^{a-d}

3.1.3 The incorporation of three consecutive α-hydroxy-β-amino acids arrests translation - Target Peptides B_1 and B_2

Next, I investigated whether the ribosome could incorporate three consecutive α-hydroxy-β-amino acids by translating the mRNAs encoding pentapeptides B_1^{a-d} and B_2^{a-d} (Figure 3.2). With the exception of peptide B_2^{b}, the pentapeptide yields for all of the mRNAs encoding three consecutive α-hydroxy-β-amino acids were ~10% or lower, compared to ~90% for the control pentapeptide containing three consecutive Phe residues. Instead, the results reveal the accumulation of either di-, tri- or tetrapeptide. In contrast, the mRNA encoding peptide B_2^{b} resulted in pentapeptide yields of ~40%.

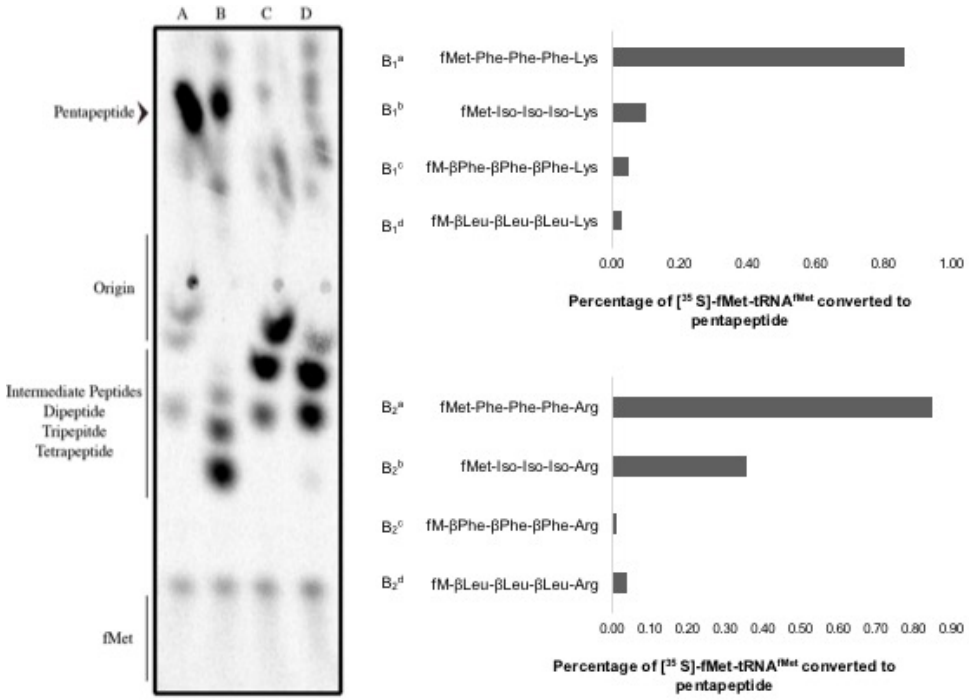


Figure 3.2 The ribosomal incorporation of three, consecutive α-hydroxy-β-amino acids

Note: The eTLC to the left represents the translations B_2^{a-d}

3.1.4 Placing α-amino acid spacers between the α-hydroxy-β-amino acids increases β-peptide yields - Target peptides C_1 and C_2 vs Target peptides D_1 and D_2

I next sought to determine whether the addition of α-amino acid spacers between the α-hydroxy-β-amino acids would improve their incorporation efficiency. The ribosomal incorporation of two α-hydroxy-β-amino acids separated by a single α-amino acid spacer is shown in Figure 3.3. The results show that for most of the pentapeptides containing a single α-amino acid spacer (C_1^b, C_1^c and C_1^d, and C_2^c and C_2^d) there was a significant reduction in full-length peptide yields (compare tetrapeptide yields in Figure 3.1 with pentapeptide yields in Figure 3.3). Interestingly, the results reveal the accumulation of a major radioactive species that appears to correspond to the formation of a tetrapeptide intermediate. This suggests that both of the α-hydroxy-β-amino acids were incorporated, but failed to function as peptide chain-donors. To ensure that the accumulated product corresponding to tetrapeptide, control translation reactions were performed with Phe-$tRNA^{Phe}$, which yielded tri-, tetra-, and penta-peptide (Figure 3.).

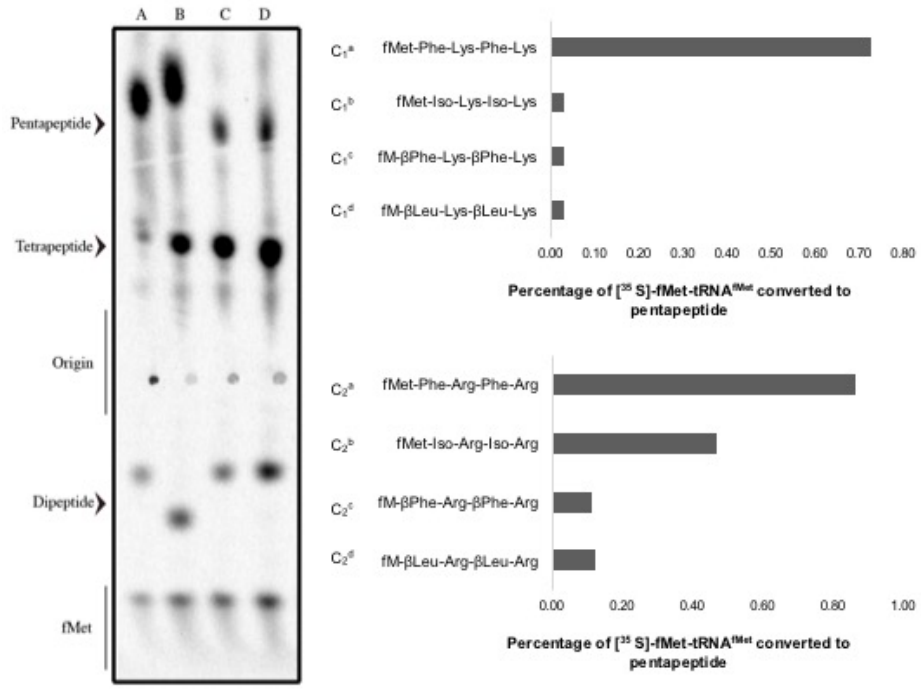


Figure 3.3 The ribosomal incorporation of two α-hydroxy-β-amino acids separated by a single α-amino acid spacer

Note: The eTLC to the left represents the translations C_2^{a-d}

The final yield for pentapeptide C_2^{b} (50%) was essentially the same as the tetrapeptide yield observed in the absence of the α-amino acid spacer.

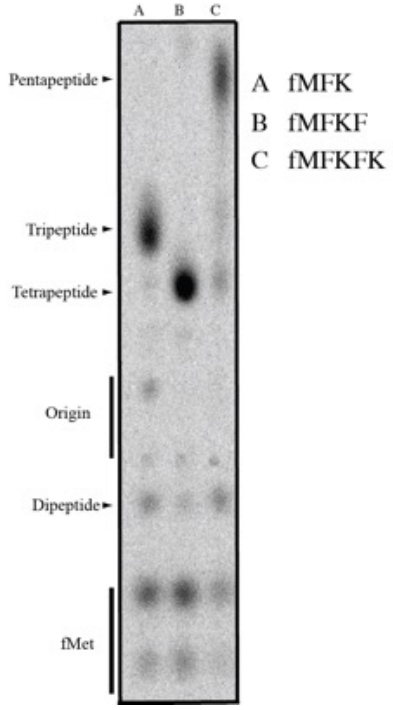


Figure 3.4 Translation of control peptides, fMFK, fMFKF, and fMFKFK

Unlike the results obtained with a single α-amino acid spacer, the addition of a three α-amino acid spacer between two α-hydroxy-β-amino acids resulted in a significant increase in the ribosomal incorporation of two α-hydroxy-β-amino acids (Figure 3.4). Relative to the yields obtained with a single α-amino acid spacer, the addition of a three α-amino acid spacer resulted in a ~10-fold increase in the full-length peptide yields for peptides $D_1^{b\text{-}d}$. The full-length peptide yields for peptides $D_2^{b\text{-}d}$ were ~1.5-fold higher than those observed for the D_1 peptides. Notably, the results were not side chain dependent.

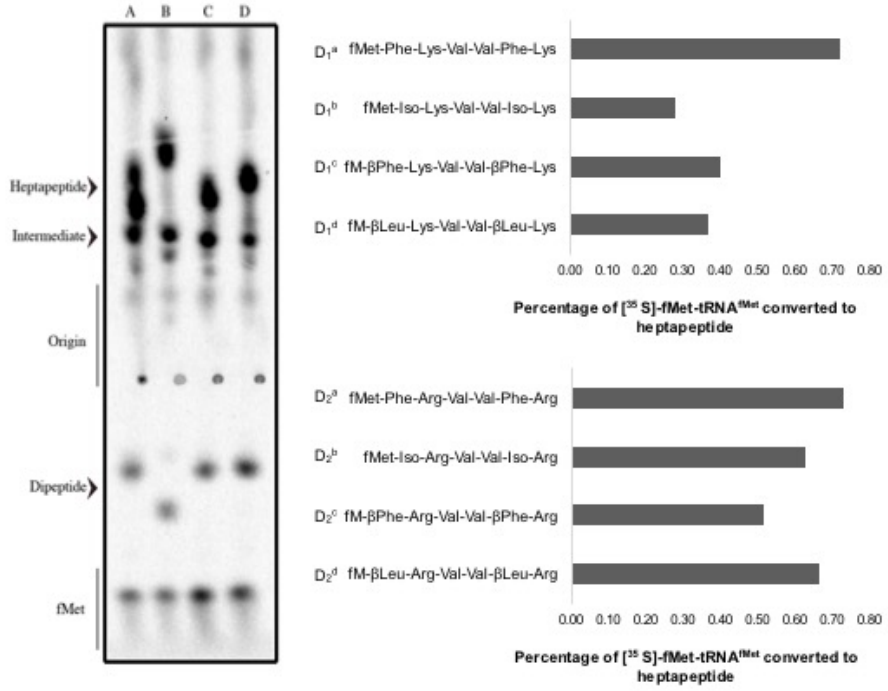


Figure 3.4 The ribosomal incorporation of two α-hydroxy-β-amino acids separated by a three α-amino acid spacer

Note: The eTLC to the left represents the translations D_2^{a-d}

Discussion

Despite the interest in β-peptides foldamers for their proteolytic stability and ability to self-assemble into secondary structures that mimic protein surfaces, their synthesis relies in SPPS, which is not practical for building diverse libraries of these peptidomimetics. We hypothesized that we could circumvent this problem by using the TM to synthesize peptides with α-hydroxy-β-amino acids. In Chapter 2, I confirmed that the TM incorporates these monomers into dipeptides and tripeptides using eTLC and HPLC β-RAM to calculate peptide yields and confirm peptide identities. In this chapter, I expanded upon the results obtained in Chapter 2 by testing the ability of the TM to incorporate multiple α-hydroxy-β-amino acids within the same peptide. By varying the identity of the charged amino acid, translating four different mRNAs and quantifying the results via phosphorimager analysis, I demonstrate that the TM can successfully synthesize peptides with two α-hydroxy-β-amino acids.

3.1.5 The ribosomal incorporation of multiple α-hydroxy-β-aminoacyl-tRNAs exhibits a side chain dependence

The most exciting finding to emerge from this study is the successful ribosomal incorporation of multiple α-hydroxy-β-amino acids within one peptide. However, it is worth noting that tetrapeptide and pentapeptide translations with Arg-tRNA^{Lys} seem to be side-chain dependent. For example, translations with mRNAs A, B, and C show that Iso, which lacks a side chain, exhibits a higher final incorporation yield than β-Phe or β-Leu. This side-chain dependent incorporation indicates that sterics influence the ribosome's catalytic activity. This concept is not new as several studies have shown that D-amino acids and other UAAs with bulky side chains are poor ribosomal substrates.[18,19] Interestingly, this side chain dependence is not observed when I translate mRNA D. This result could indicate that any deleterious steric interactions within the PTC becomes less pronounced when a three α-amino acid spacer is included between the two α-hydroxy-β-amino acids.

3.1.6 Incorporating multiple adjacent α-hydroxy-β-amino acids triggers ribosome stalling

The translation of mRNAs B and C results in a significant accumulation of intermediate product that is greater than the yields obtained for the desired full-length peptide. I interpret intermediate product accumulations as instances where the peptide structure induces ribosome stalling. While stalling was not observed in the single incorporation assays performed in Chapter 2, the multiple incorporation assay is a more stringent assay for TM activity.[20] Furthermore, it is possible that the single incorporations of α-hydroxy-β-amino acids may be affecting the PTC activity but the populations of ribosomes that are affected might be minor enough that they remain undetected. However, because the multiple incorporation assay is a more rigorous assay for the TM activity, a higher percentage of ribosomes become incompetent as a result of the multiple unnatural backbones in the PTC, making it possible to quantify peptide accumulations by eTLC. Meaning, when more than one α-hydroxy-β-amino acid is present in the PTC, any stalling events become cumulative and therefore, easier to detect. While it is difficult to characterize the nature of the stalling mechanism in this instance, we can attempt to explain the results based on previous characterizations of different stalling mechanisms, and therefore, propose experiments, to address the stalling and make this technology even more robust. Below, I will discuss the different *in vitro* stalling

mechanisms, how they relate to the multiple incorporation of α-hydroxy-β-amino acids, and propose experiments to rescue ribosome stalling, where appropriate.

There are two known triggers of ribosome stalling – the nascent peptide interacting with the exit tunnel or the nascent peptide interacting with the PTC in an unproductive manner. The exit tunnel is located in the 50S subunit and is mostly composed of 23S rRNA, although ribosomal proteins L4 and L22 are also prominent structures found in the exit tunnel's most constricted section. The tunnel is about 80-100 Å long, has a diameter that varies from 10 Å at the narrowest part and 20 Å at the widest part, and protects 30-40 amino acids of an extended polypeptide chain.[21,22] The growing polypeptide chain can adopt secondary structures similar to an α-helix as it transverses through the exit tunnel; in that case the exit tunnel can protect up to 60 amino acids.[23] The exit tunnel is also sensitive to the sequences that are translated. For instance, short peptides containing prolines provoke ribosome stalling by interacting with the exit tunnel's 23S rRNA, inducing a conformational change in the 23S rRNA backbone that restricts PTC activity.[24] Ribosome stalling is also observed in the elongation of certain peptide sequences, such as proline and glycine polypeptides, where either the A-site tRNA accommodation, peptidyl transfer, or translocation steps are affected.[25–27] Peptide chains do not reach the exit tunnel until they have reached about seven or eight amino acids in length (24.5-28Å).[28] As a result, the stalling observed with the di-, tri-, and tetrapeptides is unlikely to occur as a consequence of unfavorable interactions with the exit tunnel.

Ribosome stalling can also be triggered by premature peptidyl-tRNA drop-off which competes with translation elongation.[29] Peptidyl-tRNA drop-off events occur when the concentration of a particular aa-tRNA is low or completely absent. When aa-tRNA concentrations are low, they create 'starved codons' because ternary complex formation, its delivery to the ribosome and A-site decoding happen at a slower rate. In the case of UAA-tRNAs, the amino acid identity might affect the binding affinity for EF-Tu, which slows the rate at which they are delivered to the ribosome and thus lowers their incorporation efficiency.[30–32] Since the experiments described in this chapter are conducted with EF-Tu in 30-fold excess over the α-hydroxy-β-aminoacyl-tRNAs, stalling is unlikely to originate from slow ternary complex formation, or α-hydroxy-β-aminoacyl-tRNA^{Phe} delivery to the A-site. However, it is possible that α-hydroxy-β-aminoacyl-tRNAs bind EF-Tu in an inefficient manner, which increases the α-hydroxy-β-aminoacyl-tRNA^{Phe} A-site sampling time. Measuring the binding affinity of α-hydroxy-β-aminoacyl-tRNAs to the ribosome using filter

binding assays would provide further insights into this step.[33,34] It is also possible that the ribosomal A site discriminates against α-hydroxy-β-amino acids. Chemical probing experiments where the 23S rRNA protection patterns obtained with natural aminoacyl-tRNAs and α-hydroxy-β-aminoacyl-tRNA interactions would shed light on role of the amino acid in A-site accommodation. Furthermore, because the α-hydroxy-β-amino acid backbone is more flexible than natural amino acid backbones due to the additional carbon,[1] the conformational flexibility of α-hydroxy-β-amino acids may keep the P- and A-site substrates from adopting the correct conformation in a timely fashion. This could also manifest as the accumulation of intermediate peptide products observed by eTLC. This explanation is particularly appealing since the ribosome's main mechanism of action is lowering the activation energy of peptide bond formation by stabilizing the P- and A-site substrates in the correct conformation.[35] If P- and A-site substrate stabilization becomes difficult with the multiple incorporation of α-hydroxy-β-aminoacyl-tRNA^{Phe}s, peptidyl drop-off may compete with the elongation step.

Peptidyl drop-off can also be catalyzed by IF1/IF2 in systems where RFs are missing and there are 'starved' codons, such as this one.[36] IF1/IF2 catalyzed peptidyl drop-off is most effective when the ribosome is translating short messages that code for 5 amino acids or less,[37] this biochemical observation could provide an alternate avenue for explaining intermediate product accumulation. If the A-site and P-site tRNAs are unable to adopt the proper conformation for peptide bond formation to occur, then IF1/IF2 could catalyze peptidyl drop-off. We can test this hypothesis using nitrocellulose binding assays to determine the percentage of peptidyl-tRNAs bound to the ribosome at different stages of elongation.

Another possibility is that the O, N acyl shifts required for native peptide bond formation occur concurrently and co-translationally. If simultaneous rearrangements are incompatible with the large subunit's 23S rRNA backbone, it could lead to conformational rearrangements within the 23S rRNA backbone that render the PTC inactive, regardless of the nature of the aa-tRNA being incorporated. Thus, the multiple O, N acyl shifts could trigger ribosome stalling. A direct way to test this hypothesis would be to block the O, N acyl shift from occurring by converting the amine to an azide (α-hydroxy-β-azide) and charging it onto tRNA^{Phe} so that the only component that is different in the reactions is the aminoacyl moiety. Translating α-hydroxy-β-azide tRNA^{Phe} and releasing the peptide with puromycin (pmn), would yield an ester with azides that can be reduced with tris(2-carboxyethyl)phosphine (TCEP). The TCEP reduction

would trigger the O, N acyl shifts that result in native amide bonds *only* after all the relevant aa-tRNAs have been incorporated.

Translation stalling is mostly alleviated when mRNA D is translated. A potential explanation is that the heptapeptides containing the two α-hydroxy-β-amino acids are long enough to reach the exit tunnel (25 Å and 26.56 Å for the natural and unnatural peptides, respectively). This could result in favorable interactions with 23S rRNA that reduces any problems within the PTC. Finally, because the identity of the A-site tRNA has been implicated in ribosome stalling, it might be interesting to change the identity of the charged aa-tRNAs to another amino acid, such as alanine or glutamine, to probe the effects of the incoming aa-tRNA on α-hydroxy-β-aminoacyl-tRNA elongation.[38] This approach would be especially attractive as the identity of the A-site aa-tRNA has been implicated ribosome stalling.[38]

Conclusion and Future Directions

Taken together, these results reveal that the ribosome can synthesize peptides with two consecutive α-hydroxy-β-amino acids or two α-hydroxy-β-amino acids separated by a three α-amino acid spacer. In contrast, the ribosome is unable to efficiently synthesize pentapeptides with three α-hydroxy-β-amino acids flanked by two α-amino acids or alternating α-hydroxy-β-amino acids with α-amino acids, as observed by the intermediate peptide accumulation. Intermediate peptide accumulation is consistent with ribosome stalling due to premature tRNA drop-off, a possible consequence of multiple α-hydroxy-β-amino acids adopting incompetent conformations within the PTC that are unable to enter subsequent elongation cycles. These findings offer a starting point for ways to improve the incorporation α-hydroxy-β-aminoacyl-tRNAs and for the *in vitro* synthesis of diverse β-peptide foldamer libraries.

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4 Chapter 4 Materials and Methods

Materials

All the protocols described here were adapted from the protocols described in Dr. Phil Effraim's dissertation, and Dr. Mike Englander's and following Dr. Margaret Elvekrog's dissertation.

Tightly-coupled 70S ribosomes from MRE 600 cells were prepared as described.[1] Initiations factors, and elongation factors were previously prepared by former Gonzalez Lab and Cornish Lab members.[1] All tRNAs were purified from *E. coli* and purchased from Sigma Aldrich or MP Biomedicals ($tRNA^{fMet}$, $tRNA^{Phe}$, $tRNA^{Lys}$) mRNA templates were obtained by run-off in vitro transcription using double-stranded, linear DNA encoding variants of bacteriophage T4 gene product 32 (T4gp32) and T7 RNA polymerase.[1] All amino acids were purchased from ChemImpex. For the synthesis of di- and tripeptides, all the chemicals and solvents were purchased from Sigma Aldrich and used without further purification.

Methods

4.1.1 Enzymatic Aminoacylation of natural aa-tRNAs

All the tRNAs used for these studies were purchased from Sigma Aldrich. The ^{35}S-Met was purchased from Perkin Elmer. MTHF was prepared following Dr. Margaret Elvekrog's dissertation, section 5.1.1.1.

4.1.1.1 fMet-tRNA^{fMet}.

125 mM Tris-HCl pH 7.5 at 37C, 35 mM MgCl2, 750 mM KCl, 0.5mM EDTA, 5 mM DTT was incubated with 20 μM tRNA, 80 mM Met, 300 μM 10-FTHF, 2.5 mM ATP, 0.02 μM MetRS, and 0.2 μM transformylase for 10 min.

4.1.1.2 ^{35}S-fMet-tRNA^{fMet}

125 mM Tris-HCl pH 7.5 at 37C, 35 mM MgCl2, 750 mM KCl, 0.5mM EDTA, 5 mM DTT was incubated with 20 μM tRNA, 4 μM Hot Met, 300 μM 10-FTHF, 2.5 mM ATP, 0.02 μM MetRS, and 0.2 μM transformylase for 5 min. After 5 min, add 16 μM Met. Incubate for another 5 min.

4.1.1.3 Phe-tRNA^{Phe}

Mix15 ṵ M tRNA^{Phe};Phe 55 mM; 200 mM TrisHCl pH 7.5; 15 mM MgCl; 25mM KCl, 2 mM BME; 5mM ATP; 10 mM PEP; 30 μM pyruvate kinase; 0.75 μM Phe aaRS. Incubate at 30C for 10min.

4.1.1.4 Lys-tRNA^{Lys} and Val-tRNA^{Val}

50 mM TrisHCl pH=7.5; 7mM MgCl2; 150 mM KCl; 0.1mM EDTA; 1mM DTT; 20 μM tRNA; 2.5 mM ATP; 80 mM amino acid; 1.1 μM aaRS.

All enzymatic natural aa-tRNA reactions were Phenol extracted two times and chloroform extracted once. 0.1x volumes of 3M NaOac pH=5 were added along with 3x volumes of cold ethanol. Precipitate overnight in the -80C freezer. Spin at maximum speed for 30min at 4C. Wash pellet with 70% Ethanol. Resuspend the pellet and purify using P6 column.

Efficiency for fMet-tRNA^{fMet} and Phe-tRNA^{Phe}: determine charging efficiency by FPLC using the cold reaction (Buffer A 1.7M Ammonium Chloride, 10mM Ammonium Acetate pH 6.3; Buffer B 10 mM Ammonium acetate pH 6.3, 10% MeOH).

Efficiency for Lys-tRNA^{Lys} and Val-tRNA^{Val}: For efficiency calculations, set up a small scale reactions with ^{32}P-labeled tRNA.[2]

4.1.2 Flexizyme Charging of Unnatural Amino Acids

Flexizyme T7 Run-off Transcription was done according to published procedures. [3]

Flexizyme charging reactions. 20 μM tRNA; 600 mM MgCl2; 20 ṵ M eFx or dFx; 100 mM Hepes-KOH; 5 mM amino acid. To determine charging efficiency, follow ^{32}P-labeling protocol to label tRNA, and run a the small scale reaction with 100 pmol of tRNA final.[2] Incubate reaction mixture on ice until aminoacylation reaction is done.**

Time Course Summary of Flexizyme Charging Efficiencies onto $tRNA^{Phe}$ **

Unnatural Amino Acid	Incubation Time at 0°C
Isoserine**	2 hours
α-hydroxy-β-Azide	6 hours
α-hydroxy-β-Phenylalanine**	24 hours
α-hydroxy-β-Leucine**	24 hours
Arginine	6 hours

**Conditions determined by Miguel Jimenez.

Once ready to digest the hot reaction, incubate for 10 min at room temperature. Quench P1 digestion with 15 µL of 200 mM NaOAc pH 5. Spot 0.25µL, 0.5µL, 0.75 µL, and 1 µL of the mixture on the TLC plate. Run the plate ¾ of the way.

Work up cold reaction

Quench with 0.1x reaction volume of 0.6M NaOAc pH 5. Add 3x reaction volume of 100% Ethanol. Precipitate for 3 hours or overnight. Pellet, wash with 70% Ethanol and resuspend with 20 uL of 10 mM KOAc pH 5. For the multiple incorporation experiments, minimum of 30 ¨ M was necessary each aa-tRNA

4.1.3 Translation Buffers

Three different buffers were prepared for the translations. All buffers were filtered under steril conditions and aliquoted for single use.

5x Polymix –Mg (250 M TrisOaA, pH=7.5; 500 mM KCl; 25 mM NH4OAc; 2.5 mM Ca(OAc)2; 25 mM putrescine; 5 mM spermidine; 30 mM BME; 5% glucose)

5x Polymix + Mg (250 M TrisOaA, pH=7.5; 500 mM KCl; 25 mM NH4OAc; 2.5 mM Ca(OAc)2; 25 mM putrescine; 5 mM spermidine; 30 mM BME; 5% glucose, 15.35 mM Mg(OAc)2)

1x Buffer 6 (50 mM TrisOAc pH=7.5; 50 mM 1M NH4OAc; 100 mM KCl; 5 mM MgOAC; 0.5 mM Ca(OAc)2)

4.1.4 Translation Reactions

Final concentrations for translation components in (μM)

Tightly coupled 70S ribosomes(0.5); IF1 (0.75); IF2 (0.75); IF3 (0.75); GTP (500); Mg^{2+} (3.5); ^{35}S-fMet (.25); mRNA (3); EF-Tu (30); EF-TS (3); EF-G (1.5); aa-tRNAs (1 per incorporation)

Initiation complexes

Add ribosomes, initiation factors, GTP, polymix buffer +Mg, polymix buffer –Mg and water. Incubate at 37C for 10min. After the incubation period is over, add mRNA and water (if necessary). Incubate for 10min at 37C. Then, add ^{35}S-fMet-tRNA^{fMet} and water and incubate at 37C for 10 min. Quickly spin down and keep at 0 C for at least 10min or until you are ready to assemble complexes.

EF-G Complexes

Mix 1.5 μM EF-G, 1 mM GTP, 3 μM phosphoenol-pyruvate, and 0.001 units/μL pyruvate kinase in polymix buffer +Mg buffer and store on ice until use.

Pre-Ternary Complexes

Mix 30 μM EF-Tu, 10 μM EF-Ts, GTP, pyruvakte kinase and PEP, Polymix buffer +Mg, Polymix Buffer –Mg and Buffer 1x. Incubate at 1 min at 37C and 1 min on ice.

Ternary Complex

Assemble the necessary ternary complexes by adding 4.5 uL of preternary complex, required aa-tRNA(s), and water, if needed. Keep on ice until ready to use.

Pre-Reaction Complex

Mix 1.2 μL EF-G complex and 6.4 μL Initiation Complex. Keep on ice until ready to assemble translation reactions

Assembling Translation Reactions

Make sure all the tube contents are mixed well and spun down before starting the reaction. Incubate Pre-Reaction Complex and Ternary Complex at 37C for 5m. Mix 6.4 μL ternary complex with Pre-reaction Complex fast *as* you start the timer. Incubate for 1h at 37C.

Quenching the reactions

Quench 0.5 uL of rxn with 1 uL of 1M KOH. Mix well. Incubate at 37C for 5 min to make sure all radioactive species are released (this step minimized the origin). Then, spot 0.25 or 0.2 uL of the quenched reaction on the eTLC plate. Wait for the spots to dry. Wet the paper with running buffer without drenching the paper. If you drench the paper, your spots will not resolve. Use a long pipet to roll the buffer on the paper and roll the excess off the paper. Place the eTLC on the chamber and run at constant Volts for 30min. Take the plate out and let air dry 30-45min. Place the plate on the phosphorimager plate with saran wrap over it. Make sure that you don't move the wrap once you have place the phosphorimager plate on the cover and close it. Leave overnight.

Imaging the plate – Analysis: ImageQuant

Draw circles around each spot of interest from top to bottom. Then, draw a large square in an empty area – this will be your background. When you report the volumes, delete all the columns except Volume and Area. Normalize the intensities obtained by eTLC and calculate yields: spot/(sum spots on the same lane)*100 = percent peptide.

Authentic Marker Synthesis

Synthesis of Fmoc-amino acids for Solid Phase Peptide Synthesis

Dissolve 1 eq of amino acid in 5 mL water. Add 2 eq of $NaHCO_3$. Cool to 0°C. Dissolve Fmoc-OSu in 5 mL dioxane. Add Fmoc-OSu solution dropwise to amino acid solution. Stir at room temperature for 16 hours. Extract with 20 mL of 1:1 ether : ethyl acetate. Wash the organic layer with saturated $NaHCO_3$. Combine the aqueous layers and acidify with 1M HCl to pH 3. Extract 3x with 20mL ethyl acetate. Wash organic layers 1x with brine. Dry with sodium sulfate, decant and dry under reduced vacuum.

Solid Phase Peptide Synthesis (SPPS)

Fmoc-Lys (N-Boc) Resin Substitution: 0.43 mmol/g	
1 equivalent 0.1 g of resin (limiting factor)	0.04 mmol
4 equivalents (uaa)**	0.17 mmol
10 equivalents (for nat aa)	0.43 mmol
9.9eq (for HCTU)	0.43 mmol
20 eq for DIPEA	0.86 mmol

* Prepare 0.5M for amino acids

Swell resin with 3 mL of dichloromethane. Deprotect resin for 30m with 3 mL of 20% piperidine in NMP. Wash resin with 5x5mL of NMP. Add the Fmoc-Phe, HCTU and DIPEA for the first coupling for 1 hour. Deprotect the resin with 3mL of 20% piperidine in NMP for 30m. Wash resin with 5x5mL of NMP. Couple fMet (add fMet, HCTU and DIPEA) for one hour. Wash resin with 5x5 mL NMP, 5x5 mL Methanol 5x5 mL Dichloromethane, and 5x5 mL diethyl ether. Keep under house vacuum for 10 min to remove any residual solvent. To cleave resin, add 1 mL of 95% Trifluroroacetic Acid (TFA), 2.5% Triisopropyl Silane, 2.5% water. Stir for 4 hours. Note that sometimes solutions turn pink. After 4 hours, tightly plug a 1mL glass pipette with glass wool and filter off the resin. Add 15 mL of chilled diethyl ether (you will see a precipitate form). Centrifuge for 20min at 4°C. This will pellet the precipitated peptide. Decant Ether and purify by HPLC.

Dipeptide Synthesis

Boc-amino acid Synthesis

Dissolve 1 eq of amino acid in 5 mL water. Add 3 eq of triethyl amine. Cool to 0°C. Dissolve Boc_2O in 5 mL dioxane. Add Boc_2O solution dropwise to amino acid solution. Stir at room temperature for 16 hours. Add 1M NaOH to pH 10. Wash aqueous layer 2x with 20 mL ether. Discard organic layer. Acidify with 1M HCl

to pH 3. Extract aqueous later 3x with 20 mL ether and combine these organic layers, wash 1x 20 mL brine. Dry with sodium sulfate, decant and dry under reduced vacuum.

Benzyl ester Boc amino acid Synthesis

Under argon: Add 1.2 equivalents of Boc-amino acid, 1equivalent of benzyl bromide, 2 equivalents of anhydrous triethyl amine and 3 mL of anhydrous DMF and stir overnight at room temperature under argon. Dilute reaction with ether and wash 3x with 10 mL 0.5M HCl, 3x with 10mL saturated $NaHCO_3$, and 1x 20mL brine. Dry with sodium sulfate, decant and dry under reduced vacuum.

TFA Deprotection Benzyl ester Boc amino acid

To Benzyl ester Boc amino acid, add 5 mL dichloromethane. Cool to 0°C and add 5 mL TFA. Stir at 0°C for 1 hour. Dry under vacuum and purify by flash column chromatography using a 0-15% methanol in dichloromethane gradient.

Coupling fMet to Benzyl Ester amino acid

Under argon, add 1 equivalent of fM, 1.2 equivalents of Benzyl ester amino acid, 1.1 equivalents of EDC·HCl, 4.33 equivalents of DIPEA, and 3 mL DMF. Stir under argon at room temperature overnight. Remove DMF under reduced vacuum and purify by flash column chromatography using a 0-15% methanol in dichloromethane gradient.

Deprotection fMet-amino acid benzyl ester

Under Argon: To fMet-amino acid benzyl ester, add 0.1 equivalents of Pd/C, and 3 mL anhydrous Methanol. Then, add H_2(g). Stir overnight under H_2(g).Add Methanol and filter over celite to remove Pd/C and purify by flash column chromatography using a 0-5% methanol in dichloromethane gradient. Purify by HPLC.

HPLC crude di- and tripeptides: monitor 220 nm

Dissolve crude peptide in 5mL of water with 0.1% TFA and filter through 0.2 micron filter

Peptide	HPLC Purification Conditions
fM-F	0%-40% Acetonitrile over 80min
fM-F-K	0%-40% Acetonitrile over 80min
fM-Iso	0%-40% Acetonitrile over 80min
fM-Iso-K	0%-20% Acetonitrile over 40min
fM-βPhe	0%-40% Acetonitrile over 80min
fM-βPhe-K/fM- βPhe-R	0%-40% Acetonitrile over 80min
fM-βLeu-K	0%-40% Acetonitrile over 80min
fM-βPhe-βPhe-K	0%-50% Acetonitrile over 25min
fM-βPhe-K-βPhe-K	0%-60% Acetonitrile over 30min
fM-βPhe-βPhe-βPhe-K	0%-80% Acetonitrile over 40min
fM-βPhe-K-V-V-βPhe-K	0%-90% Acetonitrile over 45min

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